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Morphologic Lesions, Hematological and Immunological Responses and Neutrophil Functions in Fetal, Neonatal and Adult Sheep Infected With Brucella Abortus.

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MORPHOLOGIC LESIONS, HEMATOLOGICAL AND IMMUNOLOGICAL RESPONSES AND NEUTROPHIL FUNCTIONS IN FETAL, NEONATAL AND ADULT SHEEP INFECTED WITH BRUCELLA ABORTUS

The Louisiana State University and Agricultural and Mechanical Col. PH.D. 1983

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MORPHOLOGIC LESIONS, HEMATOLOGICAL AND IMMUNOLOGICAL RESPONSES AND NEUTROPHIL FUNCTIONS IN FETAL, NEONATAL AND ADULT SHEEP INFECTED WITH *BRUCELLA ABORTUS*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
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in partial fulfillment of the
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Doctor of Philosophy

in

The Interdepartmental Program
in Veterinary Medical Sciences
Veterinary Pathology

by
Sammy Lee Gorham
D.V.M., Tuskegee Institute, 1978
B.S., Tuskegee Institute, 1976
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ABSTRACT

Fetal, neonatal and adult sheep were infected intramuscularly with B. abortus Strain 2308. Morphologic lesions were present in the regional lymph nodes of B. abortus infected neonatal and adult sheep and in the regional lymph nodes, lungs and the parietal surfaces of thoracic and abdominal organs of B. abortus infected fetuses. The regional lymph nodes of neonatal and adult sheep were moderately to markedly enlarged and contained prominent lymphoid follicles and few to numerous plasma cells. Fetal lesions were characterized by: lymph nodes with sinuses that contained numerous foamy macrophages, pneumonia and fibrin covered thoracic and abdominal organs.

Fetuses responded to B. abortus infection with a neutrophilia, lymphopenia and occasional monocytosis. Neonatal and adult sheep did not have significant hematological responses to B. abortus infection. An antibrucella antibody response was not present in fetuses but adult and neonatal sheep produced brucella specific antibodies as early as PI day 8. Fetal neutrophil phagocytic but not killing function against B. abortus and S. aureus was enhanced by B. abortus infection. Neonatal phagocytic and killing functions against B. abortus were increased by B. abortus infection while only phagocytic function against S. aureus was increased. Adult sheep

phagocytic and killing functions were not altered by B. abortus infection. Fetal serum cortisol levels were markedly elevated by B. abortus infection.

B. abortus Strain 2308 produced morphologic lesions, hematological, immunological and cortisol responses and increased neutrophil functions in sheep. Because most of these changes are similar to those reported in cattle, sheep, especially during fetal development, could serve as an experimental model for bovine brucellosis.

Chapter I

Literature Review and Objectives

Introduction

Brucellosis is a highly infectious disease of domestic animals and man caused by members of the genus Brucella. In animals, brucellosis is characterized by abortion, reduction in fertility, decreased milk production, orchitis and epididymitis.[1-6] Human brucellosis is a severe disease manifested by muscle pain, weakness, sweating, chills and fever.[7,8]

Brucella infections are primarily spread by infected animals that excrete brucella organisms in genital secretions shortly before or after abortion or parturition or in colostrum or milk. The primary portals of entry are the oral mucosa of neonates drinking infected milk and the conjunctiva and nasopharynx of animals exposed to infected genital secretions.[3,9,10] Humans are infected by consuming raw milk, dairy products and meat products infected with Brucella spp.. Brucellosis is an occupational disease for veterinarians, meat inspectors, livestock and packing plant employees and other people who work with infected animals.[7,8,11]

The Genus Brucella

The 6 species of the genus Brucella which have been identified are B. melitensis, B. abortus, B. suis, B. ovis,

B. canis and B. neotomae. These organisms tend to localize and frequently persist in lymphoid organs, fetal and placental tissues, seminal vesicles and testes.[1-6,12] They are pathogenic for several mammalian species. B. melitensis primarily affects goats and sheep while B. suis is a swine pathogen. Cattle are most susceptible to B. abortus infection and dogs are principle hosts for B. canis infection. B. ovis infections are limited to sheep and B. neotomae is primarily pathogenic for rodents. All Brucella spp. have the ability to produce abortion, orchitis and epididymitis; all with the exception of B. ovis are capable of causing a variety of symptoms in several species, including man.[1,3,12]

History

The first member of the genus Brucella was isolated in 1887 by David Bruce[13], an officer of the British Army Medical Staff. He cultured what he called "micrococci" from spleens of British soldiers who died from Malta fever. This organism was not given a formal name until 1892 when Hughes, a British army officer, called it Streptococcus miletensis.[14] Subsequently, the genus name was changed, but the species name was retained (the spelling was changed). By 1893 the organism was referred to as Micrococcus melitensis.[14]

In 1897, Bang, a Danish veterinarian, isolated the causative organism of bovine contagious abortion from an aborted bovine fetus.[15] Because some of these organisms

were elongated, he called them bacilli. He named this bacterium "Bacillus of abortion." During the next 20-30 years this organism was called Bacterium abortus and Bacillus abortus.[14]

Traum isolated Bacterium abortus from an aborted pig fetus in 1914.[16] In a subsequent isolation of Bacterium abortus from aborted pig fetuses, it was inferred that this organism was different from the Bacterium abortus commonly isolated from aborted bovine fetuses.[17]

In the 1920's Evans placed the organisms originally isolated by Bruce, Bang and Traum in the genus Brucella. Respectively they were named Brucella melitensis, Brucella abortus and Brucella suis.[12] Brucella ovis was isolated from sheep in Australia in 1953[18] and Brucella canis was isolated from aborted canine fetuses in the United States in 1966.[19]

Morphology, Cultural Features and Biochemical Reactions

Brucellae are short rods or cocco-bacilli, Gram stain negative, facultative, intracellular bacteria. They are arranged singularly or in short chains. Organisms are nonmotile and have poorly developed capsules. Spores are not formed.[3,12]

All Brucella species have rough and smooth strains. Rough strains are viable mutants of smooth strains. They lack part of the polysaccharide side chain of the lipopolysaccharide (LPS) present in the outer wall of Gram negative bacteria.[3,12] This mutation in the LPS causes

rough strains to be more permeable to lysozymes and antibiotics than smooth strains[12] and potentially decreases the pathogencity of rough strains.

Brucella spp. can be isolated and grown on artificial media with little difficulty. Cultivation of Brucella spp. can be obtained on Tryptose agar, Albumin agar or Trypticase Soy agar enriched with 5-10 percent heat-inactivated horse serum. Growth is barely perceivable at 2 days with maximum growth obtained between 5-7 days. Colonies are round to convex and honey colored.[3,20]

Only minor differences are detectable in biochemical reactions of the 6 Brucella spp.. Some strains of B. ovis and B. abortus require increased CO_2 atmospheric concentration to allow growth on primary isolation. This is not required of the other Brucella spp..[3,12] All species except B. ovis are capable of reducing nitrate. B. abortus is the only species capable of producing hydrogen sulfide. Oxidase production is a common feature of all species except B. ovis and B. neotomae.[3,12]

Epidemiology of Bovine Brucellosis

Bovine brucellosis is generally recognized as a worldwide problem. Thimm and Wundt (1976) stated that brucellosis was present in 153 countries. In brucellosis free countries the disease has been eradicated or never reported. In some island countries with small cattle populations, the disease has never been introduced.[22]

In the United States and probably in other countries,

cattle are the primary reservoir of B. abortus. The primary source of spread is a recently infected animal or a carrier.[22]

Brucella abortus is eventually shed with the conceptus, or in colostrum and milk after abortion or parturition. Genital excretions are the primary source of infection. Calves from infected dams may be infected in utero or shortly after birth via colostrum or milk. Most of these calves rid themselves of the infection before adulthood. However, a few do not eliminate the B. abortus infection and begin to excrete organisms shortly before or after parturition or abortion (latently infected animals).[23,24] Other means of spread reported are: infected bulls by natural service, artificial insemination with infected semen and contaminated milking machines.[3] However, spread by natural service has not been well documented.

Pathogenesis and Lesions of Bovine Brucellosis

After penetration of the mucosa following ingestion or through broken skin or the reproductive tract, B. abortus organisms localize in the regional lymph node where they enter cells, proliferate and cause chronic lymphadenitis. Small accumulations of epithelioid cells, granulomas, some with necrotic centers, and prominent lymphoid follicles develop in regional lymph nodes.[1,4]

A bacteremia develops and organisms localize in the reticuloendothelial system, udder, supermammary lymph nodes,

pregnant uterus and placenta.[4] In guinea pigs, during the bacteremic phase, organisms are ingested by von Kupffer cells and neutrophils. Neutrophils aggregate and often become trapped in hepatic sinusoids where some neutrophils and brucella organisms are ingested by reticuloendothelial cells.[25] The infiltration of macrophages was responsible for granuloma formation. A similar pathogenesis may be responsible for granulomas occasionally observed in the spleen, lymph node and liver of infected cattle.[4,26,27]

An interstitial metritis develops after bacterial localization within the uterus and uterine glands.[4] This often progresses to a severe and extensive ulcerative endometritis. At the time of abortion, large areas of the endometrial mucosa become eroded.[4] From the uterus, bacteria invade the chorionic epithelium, fetal blood vessels, allantochorionic connective tissue and fetal fluids.[4,28]

When the B. abortus infected amnionic fluid is aspirated by the fetus, it induces a bronchopneumonia.[1,4,28] Peribronchial areas and bronchioles contain fluid and fibrin admixed with predominately mononuclear cells and a few immature neutrophils.[1,4,28] The presence of a diffuse meningitis, focal interstitial nephritis, moderate subcutaneous edema and the isolation of B. abortus from several organs is evidence of fetal bacteremia.[1,3,4,28]

Brucella abortus Infection in Sheep

Sheep may be naturally or experimentally infected with B. abortus.[30-38] Natural infections are usually acquired from infected cattle.[30,32,33,34] In several experiments, B. abortus infected pregnant sheep aborted, had stillbirths or normal parturitions.[1,30,31-34,26,38] Although the ewes excrete B. abortus after abortions or stillbirths, there is little horizontal transmission within sheep flocks.[32,33,37] Latent infections in sheep have not been reported.

In an experimental study of B. abortus infection in pregnant ewes, changes were related to the stage of gestation at the time of inoculation.[37] Ewes inoculated orally with B. abortus at 135 days of gestation gave birth to uninfected twin lambs, despite isolation of organisms from the placenta and vaginal secretions. Two ewes were inoculated orally with B. abortus at 121 days of gestation. One gave birth to a live lamb 23 days later. The other ewe gave birth to a dead B. abortus infected lamb 23 days post inoculation. The vaginal secretions, placenta and milk were B. abortus positive in both ewes. An ewe inoculated with B. abortus at 98 days of gestation aborted B. abortus infected twin fetuses 25 days later while an ewe inoculated with B. abortus at 103 days of gestation gave birth to weak, B. abortus infected twin lambs 34 days after inoculation.[37]

Lesions produced in B. abortus infected fetal sheep and

B. abortus infected sheep placentas have not been well characterized. Fibrin covered abdominal and thoracic viscera were observed in twin lambs less than 24 hours old, 34 days after infection of the pregnant ewe.[37] In infected placentas, lesions were characterized by necrosis and neutrophilic infiltration of placental villi. The chorioallantoic membrane was necrotic and infiltrated by neutrophils.[36]

Adult and Neonatal Humoral Responses

Cattle with brucellosis or vaccinated with B. abortus Strain 19 vaccine produced at least 2 classes of immunoglobulins, IgM and IgG. IgM was the first antibody produced.[22,39] IgG appeared later than IgM but it persisted longer. Both IgG_1 and IgG_2 subclasses were produced.[22]

Neonatal calves (2 weeks old) and lambs (1 day old) lacked the ability to respond to several antigens[40,41,42], one of which was B. abortus Strain 19.[40,41,42] When neonatal calves and lambs were infected with B. abortus Strain 19, they responded with poor humoral immune responses.[40,41] However, at 2-4 months of age, calves responded with high levels of antibodies to B. abortus Strain 19 vaccine.[41,43] These antibody levels gradually declined and by 6-8 months after vaccination, IgG antibody was rarely detected in the blood.[43].

When ewes were inoculated with 10^{10} killed B.

abortus organisms they responded with high antibody titers within 2 weeks.[40] Neonatal lambs and fetuses inoculated with similar doses of killed B. abortus responded slowly with low levels of antibody. Anamnestic antibody responses in late term lamb fetuses and neonates inoculated with killed B. abortus organisms were as great as the anamnestic response in ewes inoculated with killed B. abortus organisms.[40]

Fetal Humoral Immune Responses

Fetal age as well as type and amount of antigen influence the production of antibody. As the fetus matures, its ability to produce immunoglobulins and antibodies increases. Immunoglobulin G has been found in 56-day-old fetal sheep[44] and IgM in 60-day-old fetal sheep[45]. At term, IgG levels were found not to exceed 22 mg/ml and IgM levels did not exceed 21 mg/ml.[44]

The first immunoglobulins produced by fetuses are IgM followed by IgG.[45-47] Ovine fetuses produced neutralizing antibodies to ØX174 phage at 41 days, to ferritin at 56 days[47,48] and chicken red blood cells at 58 days.[46,49] Antigen specific IgM and IgG were observed at 91 and 80 days of gestation[50] and neutralizing antibodies were reported in 95-100 day old ovine fetuses inoculated with bluetongue virus.[51] IgM and IgG complement fixing antibodies were observed at 123 days of gestation.[52] One study showed that anamnestic antibody responses in fetuses were as high as anamnestic

antibody responses in adults.[40]

Fetal lymphoid tissues are also reactive to certain antigens. Fetal sheep inoculated with bluetongue virus at 80 days of gestation produced mononuclear accumulations in lymph nodes. Germinal centers and plasma cells were observed at 80-85 days, the same time that IgM and IgG were demonstrated.[51] Bovine fetuses inoculated with calf rotavirus at 63 days of gestation developed prominent lymphoid follicles and diffuse pulmonary lymphoid proliferation.[53] Similar findings have been reported in ruminants inoculated with several infectious agents.[51,54,55,56]

Despite the ability to respond to a number of infectious and noninfectious agents, fetal sheep are basically agammaglobulinemic prior to 120 days of gestation.[57] There is an inability to produce antibodies to certain antigens before this gestational age. This has been associated with the delayed development of T lymphocytes to certain antigens.[57,58] It has been suggested that B lymphocytes are mature and functional at midgestation but are unable to respond because T helper cells have not matured.[44] By 120 days of gestation in the sheep, there are mature T helper cells to most antigens.[57]

Brucella Serologic Tests

The early appearance of IgM and the later appearance and persistence of IgG are frequently employed in specific

diagnostic tests for brucellosis. The most commonly used serologic tests are the card test (buffered brucella antigen), rivanol, 2-mercaptoethanol and complement fixation tests.[22,25]

The card test is a simple, rapid test used primarily to detect IgG antibiodies, however the test also detects some IgM antibiodies specific for B. abortus.[25,59] It is positive in both early and chronic reactors. In this test, suspect serum is mixed with brucella buffered antigen for 4 minutes. Agglutination is evidence of a positive reactor. Because IgM uncommonly agglutinates nonspecifically with brucella antigens in B. abortus negative or vaccinated animals and because IgG agglutinates specifically with brucella antigens, IgM is eliminated in some brucella serologic tests. In the 2-mercaptoethanol test IgM is degraded and in the rivanol test it is precipitated. In both tests the remaining IgG and brucella antigens are mixed. There is agglutination in positive reactions.[22,60] The complement fixation test is a sensitive test capable of detecting both IgM and IgG. It is frequently used to confirm a diagnosis of brucellosis when one or more of the other supplemental tests (card, rivanol, 2-mercaptoethanol) is positive.[61]

Hematology

Few studies on normal fetal hematology have been reported.[40,62] Studies on fetal hematologic responses to antigenic stimulation are even rarer.[40] Fetal sheep

inoculated with Salmonella polymeric flagellin (POL) developed a leukocytosis with lymphocytosis and neutrophilia.[40] Adults and neonates frequently respond to a wide spectrum of antigens with leukocytosis and neutrophilia.[63]

Leukocyte Function in Brucellosis

Leukocytes function in nonspecific host resistence by phagocytosing and killing bacterial organisms. Serum alone has the ability to kill certain bacteria.[64,65] However, the primary antibacterial function of serum is to opsonize bacteria so they can be ingested by neutrophils and macrophages. Opsonization by antibodies and/or antibodies and complement is essential for ingestion of most bacteria.[65-67]

An array of leukocyte function tests have been used in an attempt to determine the phagocytic and killing potentials of leukocytes for different types of bacteria.[64-66,68-75] Extracellular bacteria were more readily phagocytosed and killed than obligate intracellular bacteria.[70] Obligate intracellular bacteria often escape being killed by leukocytes. The escape from leukocyte killing may be accomplished by a variety of mechanisms. Bacterial organisms may inhibit chemotaxis, leukocyte degranulation or the leukocyte oxidative burst. Bacteria may also avoid being killed in the phagosome by being resistant to granule components or being resistant to oxidative attacks.[67] Regardless of bacterial

classification as an extracellular or an obligate intracellular parasite, the elimination of the bacteria from the host will be dependent in part on nonspecific host defenses, primarily neutrophils and macrophages.

Several studies in guinea pigs, rats and humans on leukocyte functional abilities against smooth and rough strains of B. abortus concluded that rough strains were more easily phagocytosed and killed than smooth strains.[68,70,72] Macrophages from immune rats and guinea pigs had greater phagocytic and killing potentials than macrophages from nonimmune rats and guinea pigs.[68,71,72] This may explain why immune animals are more resistent to B. abortus infection than nonimmune animals.[68.70,73] Serum had no noticable positive affect on leukocyte phagocytic and killing potentials.[62]

Neutrophils may have a major role in host defense in B. abortus infection. In nonimmune animals, neutrophils are more destructive to B. abortus than macrophages.[70] The intracellular survival of B. abortus in neutrophils from immune animals was not significantly different from survival of B. abortus in neutrophils from nonimmune animals[69]

Objectives

The objectives of this study were: 1) to evaluate the sheep as an experimental model for bovine brucellosis by examining morphologic, hematologic and immunologic responses in fetal, neonatal and adult sheep infected with B. abortus and, 2) to determine if the phagocytic and killing abilities

of neutrophils from fetal, neonatal and adult sheep are altered by B. abortus infection.

Chapter II

Morphologic Lesions, Hematological, Immunological and Cortisol Responses and Neutrophil Functions in Fetal Sheep Infected with Brucella abortus

Introduction

Fetuses possess the ability to respond to both infectious and noninfectious agents.[40,46-56,77,78] These responses are morphological, immunological and hematological. A few of these parameters have been examined in fetuses exposed to some infectious organisms. Only morphological responses have been extensively studied in fetuses infected with Brucella abortus.

Fetuses readily develop histologic and occasionally gross lesions when exposed to certain antigens.[4,18,28,37,51,56,78,79] When 90 day old fetal monkeys (gestational period of 155-165 days) were inoculated with turpentine they produced locally severe inflammatory responses.[78] Histologic lesions in fetuses exposed to various infectious agents are characterized by lymphoreticular hyperplasia[1-4,56], necrosis[79] and mononuclear inflammatory reactions.[1-4,28,51] Histologic lesions in B. abortus infected aborted bovine fetuses are characterized by bronchopneumonia, lymphoreticular hyperplasia, multiple necrotic foci in several organs and a diffuse meningitis.[1,4,28]

As the fetus matures, so does its ability to produce immunoglobulins and antibodies. Fetal sheep were capable of producing IgM antibody at 75 days of gestation (gestational period of 145-150 days) to polymeric flagellin (POL).[46] Immunoglobulin G antibody to B. ovis was evident at 80 days of gestation.[50] Neutralizing antibodies to bluetongue virus developed at 95 days of gestation[51] and IgM and IgG complement fixing antibodies to a mixture of ovalbumin, ferritin and bacteriophage were first observed at 123 days of gestation.[52]

Few hematological studies in the ovine fetus have been done.[40,62] Studies of ovine fetal hematological responses to antigenic stimulation are even rarer.(40) When fetal sheep were inoculated with Salmonella polymeric flagellin (POL.) they developed leukocytosis and neutrophilia. In 120-day-old POL inoculated fetal sheep, there was a 2-3 fold increase in lymphocytes, with maximum levels obtained within 4 days of inoculation. In 86 day old POL inoculated fetal sheep, neutrophil percentage increased from 3-6 percent to 21-25 percent of the total count within 4 days of inoculation. Percentage of neutrophils returned to normal within 12 days of inoculation.[40]

Since B. abortus was originally isolated from an aborted bovine fetus by Bang in 1897[15] there have been numerous reports of late term abortions in pregnant cattle infected with B. abortus.[28,29] These abortions probably resulted, in part, from the inability of fetal hormones to

maintain pregnancy.[80]

The objectives of this study were: to evaluate the fetal sheep as an experimental model for fetal bovine brucellosis and to determine the fetal response to B. abortus infection. These objectives were met by studying gross and histologic lesions, hematologic, immunologic and cortisol responses and neutrophil functions in fetal sheep infected with B. abortus Strain 2308.

Materials and Methods

Experimental Design -- All fetal lambs used in this study were inoculated between 116-124 days of gestation. Two fetal lambs were uninoculated controls, 3 fetal lambs were saline inoculated controls and 11 fetal lambs were inoculated with B. abortus Strain 2308.

Saline inoculated fetuses were collected at postinoculation (PI) days 4 and 8 and B. abortus infected fetuses were collected at PI days 1, 2, 3, 4 and 6.

Animals -- A total of 12 pregnant crossbred ewes were obtained from the Louisiana State University sheep farm. Animals were housed in an enclosed barn, 4 per stall. They were fed hay and tap water. All animals were negative for antibodies to Brucella abortus with the card test (buffered brucella antigen) and complement fixation test.

Ewes were bred so gestational periods could be estimated within 2 days. Pregnancy was determined by gross

enlargement of the udder and abdominal ballotment. Two fetal lambs from 2 ewes were used as uninoculated controls, 3 fetal lambs from 2 ewes were used as saline inoculated controls, and 11 fetal lambs from 7 ewes were inoculated with B. abortus Strain 2308 (Fig 1).

Fetal Inoculation -- Surgery was performed on ewes between 116 and 126 days of gestation. Water and feed were removed 8 and 24 hours prior to surgery. Ewes were preanesthetized with 10 percent surital[a] and intubated. Surgical anesthesia was maintained with metofane[b]. Midline abdominal incisions were made through the skin, linea alba and peritoneum. The head of the fetus was palpated. Inoculation of 0.5 ml of sterile saline or B. abortus Strain 2308 (3×10^3 - 4×10^6 organisms suspended in 0.5 ml sterile saline) was through the uterine wall into the dorsal muscles of the fetal neck. The incisons in the peritoneum and linea alba were sutured with 2-0 gut. The skin incision was sutured with sterile vetafil.

Fetuses were harvested by caesarean section at PI days 1, 2,3, 4, 6, and 8 immediately following euthanasia of the ewes. A few B. abortus infected fetuses were dead at time of collection and one was born alive. Sections of the lung, liver, spleen, kidney, thymus, intestinal lymph node,

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[a]Bio-centic Laboratories, Inc., St. Joseph, Missouri 64504

[b]Pitman Moore, Inc., Washington Crossing, New Jersey 08560

cervical lymph node and adrenal gland were fixed in 10 percent formalin at 4° C. Lung, liver, spleen, blood, kidney, intestinal lymph node, cervical lymph node, stomach contents, blood and amnionic fluid were collected for culture. Blood was collected via heart puncture from each fetus into sterile tubes containing the anticoagulant dipotassium ethylenediaminetetraacetate (EDTA) or in siliconized glass tubes. Blood collected in EDTA was used in leukocyte function tests, to obtain total leukocyte counts and to make blood smears. Serum from blood collected in siliconized glass tubes was used for cortisol and antibody level determinations and as autologous serum in leukocyte function tests.

Culture Procedures -- Selected tissues including lung, kidney, liver, spleen, intestinal lymph node, cervical lymph node, abomasal fluid and amnionic fluid were collected using aseptic techniques. Tissues were cultured immediately or stored overnight at 4°C and cultured within 24 hours of harvest.

Tissues were cut into 2-3 mm pieces with a sterile surgical blade. Tissue were placed in a sterile blender with 10 to 30 milliliters (ml) of sterile saline. The tissue saline mixture was blended for 20-30 seconds. Minced tissues were streaked on Tryptic Soy agar plates[C] with 5 percent heat-inactivated horse serum and brucella selective

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[C]Difco Laboratories, Detroit, Michigan.

media[81] (with the addition of 0.2 grams L cysteine, 1:5000 and 0.25 gm erythritol, 1:4000 per liter). Plates were incubated for 2-5 days in a 37° C incubator.

B. abortus was identified as small, round, pale-honey colored colonies. These bacteria were coccobacilli, Gram stain negative, urease and oxidase positive.

Bacteria -- Stock cultures of Staphylococcus aureus[d] Strain 520A were stored at 4°C on Tryptose Soy agar. For daily use, stock S. aureus was inoculated in 5-10 ml of Tryptose Soy broth incubated for 16-20 hours at 37° C. Bacteria were washed 3 times in physiologic Gey's solution, centrifuged at 1600 x g for 10 minutes at 4°C and the pellet was suspended in 5 ml of physiologic Gey's solution. The absorbance reading was adjusted to .20 - .25 optical density (OD).[e] Bacteria were cultured on Tryptose Soy agar plates for determination of the number of bacteria per ml. The microdrop plating method was used for this determination.

Brucella abortus Strain 2308[f] stock was maintained in sterile saline. Stock B. abortus was inoculated in Tryptic Soy broth and incubated at 37°C for 16-20 hours. Bacteria were washed 3 times in physiologic Gey's solution,

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[d]Dr. H. Cox, Department of Microbiology and Parasitology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803.

[e]Spectronic 20 Spectrophotometers, Bausch and Lomb, Inc., Rochester, New York.

[f]Dr. B. L. Deyoe, National Animal Disease Center, Agriculture Research Service, United States Department of Agriculture, Ames Iowa 50010.

centrifuged at 1600 x g for 10 minutes at 4° C and the pellet was suspended in 5 ml of physiologic Gey's solution. The absorbance level was adjusted to .02 - .04 OD. Bacteria were cultured on Tryptic Soy agar plates enriched with 5 percent heat-inactivated horse serum for determination of the number of bacteria per ml. The microdrop plating method was used for this determination.

Tissue Processing -- Freshly collected sections of liver, lung, spleen, thymus, kidney, intestinal lymph node and cervical lymph node were fixed in 10 percent formalin at 4° C. Tissues remained in cold formalin until processed.

Tissues were embedded in plastic as described.[82] A 5 gm pack of white powder, benzoyl peroxide (catalyst), was added to 5 g of liquid, 2-butoxy ethanol (solution B), and mixed with a magnetic stirrer. Infiltrating solution was prepared by mixing 5 ml of solution B with 50 ml of hydroxy ethyl methacrylate (solution A). Sections of tissues were added to this infiltrating solution and placed in a vacuum dessicator. A vacuum was established and maintained at 4°C for 24 hours. After 24 hours, the infiltrating solution was changed and another vacuum was created and maintained at 4°C for an additional 24 hours.

The embedding solution was prepared by mixing 50 ml of solution A with 5 ml of solution B and 2 ml of N-N dimethyl aniline (solution C). Two to three ml aliquots were pipetted into the lower depressions of plastic molds. Infiltrated tissue specimens were placed in the molds and

covered with the embedding solution and a plastic block holder. The molds were placed in a vacuum dessicator. A vacuum was created and the vacuum dessicator was maintained at 4°C for 48 hours to allow polymerization.

Using a microtome[g] with a glass knife holder, plastic embedded tissues were cut in 2 micron sections with a glass knife. Sections were stained with hematoxylin and eosin.

Hematology -- Blood was collected into EDTA tubes. The blood was used for preparation of blood smears. Total leukocyte counts were obtained with an electronic cell counter.[h] Blood smears were stained with a modified Wright's stain.[i] All blood smears were examined with an oil immerson lens for differential cell counts. Additionally, blood smears were stained for nonspecific esterase after fixation for 60 seconds in a pH 6.16 fixative composed of : KH_2PO_4, 100 mg; distilled H_2O, 30 ml; acetone, 45 ml; and formalin (30%), 25 ml.[83] Nonspecific esterase staining was obtained by incubating fixed blood smears in a solution composed of: Sorenson's phosphate buffer, 44.5 ml; hexazotized pararosaniline, 0.25 ml; and alpha-naphthyl butyrate solution, 3ml. Incubation was for 45 minutes in a 37°C water bath. Smears were washed in distilled water and counter stained in a 0.5 percent

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[g]Sorvall Porter-Blum Microtome, Ivan Sorvall Incorporated, Newtown, Connecticut 06470.
[h]Coulter Electronics Incorporated, Hialeah, Florida.
[i]Diff Quik, American Scienfic Products, Division of American Hospital Supply Corporation, McGraw Park, Il.

methyl green solution for 1 minute. Smears were then washed 3 times in distilled water. They were air dried and coverslipped. All smears were examined with an oil immersion lens for differential cell counts and nonspecific esterase positive (monocytes) and negative cells.

Neutrophil Phagocytosis and Killing Assay -- Neutrophil phagocytosis and killing functions were assayed by modifications of Pantazis' method[84] using acridine orange.[j] Blood collected in EDTA was washed 2 times in physiologic saline and once in physiologic Gey's solution followed by centrifugation at 400 x g for 10 minutes at 4°C. Approximately 0.5 x 10^6 leukocytes (in 0.3 ml of pelleted blood) were mixed with 30 x 10^6 bacteria (S. aureus Strain 520A or B. abortus Strain 2308 suspended in 0.5 ml of physiologic Gey's solution) and 0.2 ml serum (autologous or fetal calf serum) or 0.2 ml physiologic Gey's solution. Six assay mixtures prepared on each sample included: pelleted blood and B. abortus mixed with 1) autologous serum, 2) fetal calf serum, 3) and physiologic Gey's solution, and pelleted blood and S. aureus mixed with, 4) autologous serum, 5) fetal calf serum, and 6) physiologic Gey's solution. The 6 samples were incubated for 60 minutes in a 37°C water bath that rotated 200 revolutions per minute. After incubation, all tubes were vortexed and centrifuged at 400 x g for 10 minutes at 4°C. All samples were stored at

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[j]Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, New Jersey, 07410.

4° C and examined within 4 hours.

The supernatant was removed and a drop of pelleted blood and a drop of 0.14 percent acridine orange were added to a clean glass slide and coverslipped. Cells were examined with a fluorescent microscope.[k] The 63X and the 100X oil immersion lens and an epifluorescent light source with a 510mm filter were used to examine wet mounts. Three replicates of 100 leukocytes were counted for each assay mixture. Neutrophils that ingested 1 or more bacterial organisms were considered phagocytic. Neutrophils that contained 1 or more dead (red) bacterial organisms were counted as neutrophils which had killed bacteria.

Calculations of phagocytic and killing functions were obtained using the formulas:

$$\text{Phagocytosis \%} = \frac{\text{neutrophils ingesting 1 or more organisms}}{\text{total neutrophils counted}} \times 100$$

$$\text{Killing \%} = \frac{\text{neutrophils containing 1 or more dead organisms}}{\text{total neutrophils ingesting bacteria}} \times 100$$

Cortisol Assay -- The Coat-A-Count Kit[l] was used to assay fetal serum cortisol levels. The kit contained anticortisol antibody coated tubes, buffered I^{125} labeled cortisol, logit log graph paper and cortisol

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[k]Carl Zeiss, D7802 Oberkochen, West Germany.
[l]Diagnostic Products Corporation, 5700 West 96th Street, Los Angeles, California 90045.

calibrators (6 vials, A-F). A cortisol calibration curve was developed using human serum. The samples used for standardization contained 0, 1, 5, 10, 20 and 50 ug cortisol/dl.

In this cortisol assay, I^{125} cortisol competes for sites on cortisol specific antibody immobolized to the walls of polypropylene tubes. A negative control with plain tubes and a positive control with anticortisol antibody were used in this test. Twenty-five ul of the zero cortisol level was added to cortisol antibody coated tubes (maximum binding tube) and to plain tubes. Twenty-five ul of each remaining standard and 25 ul of fetal sheep serum were added to cortisol antibody coated tubes. One ml of I^{125} cortisol was added to each tube. All tubes were incubated for 45 minutes at 37°C. All tubes were inverted for 2-3 minutes to allow draining. All tubes were counted in a gamma counter[m] for 1 minute.

The percent bound cortisol was determined by the formula:

$$\text{Percent bound} = \frac{\text{net count} \times 100}{\text{net maximum bound count}}$$

The percent bound was plotted on the vertical axis of the logit log paper against cortisol concentrations on the horizontal axis for each of the calibrators (B-F). A

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[m]Searle Analytic Incorporated, 200 Nuclear Drive, Des Plaines, Illinois 60018.

straight line was drawn through the 5 points. Fetal serum cortisol concentrations were estimated by line interpolation.

Immunoglobulin Determinations -- Immunoglobulin levels, IgM, IgG_1, and IgG_2 were assayed by the Mancini technique.[85] Specific antibovine IgM was produced in rabbits. It was affinity column purified. Antibovine IgG_1 and IgG_2 were produced in rabbits.[n]

Serology - The enzyme-linked immunosorbent assay (ELISA) and an isotype specific ELISA for IgM class antibody, both with B. abortus antigen were performed.[7,8] The IgM ELISA had an affinity column purified specific antibovine IgM produced in rabbits. In the ELISA, rabbit antibovine IgG_1 and IgG_2 were used.[n]

The card (buffered brucella antigen)[14] and the complement fixation test were also used to detect brucella specific antibodies.[14,61]

Results

Animals -- After fetal inoculation with B. abortus most ewes remained in good health and clinically normal. When twin B. abortus infected fetuses 143 (1) and 143 (2) were collected the ewe was severely depressed and recumbent. At

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[n]Performed by Dr. Klaus Nielsen, Texas A&M University.

the time of collection, fetuses were viable, dead or had been born alive (Table 1).

Culture -- In all B. abortus infected fetuses, B. abortus was isolated from all cultured tissues, lymph node, spleen, kidney, liver, lung, blood, abomasal and amnionic fluid. All uninfected fetuses were B. abortus culture negative.

Pathology -- Macroscopic pathologic alterations were not observed in noninfected fetuses. All noninfected fetuses had wet, glistening organs. The lungs were firm and bright red (Fig 1). The pleura of the lung was transparent.

Gross lesions were not observed in the B. abortus infected fetus at PI day 1.

Gross lesions in the B. abortus infected fetus at PI day 2 fetus were limited to the lungs and cervical lymph nodes. The lung pleura was slightly thickened and pale white. Pleural blood vessels and lymphatics were prominent and there was slight perivascular edema. The cervical lymph nodes were slightly enlarged.

Similar gross lesions were present in all 3 B. abortus infected fetuses at PI day 3. In all 3 fetuses, gross lesions were present in the lungs and lymph nodes. The lung pleura was pale white with prominent blood vessels and lymphatic vessels. These prominent vessels were circumscribed by a white opaque fluid (edema) (Fig 2). Several 2-3 mm hemorrhagic foci were randomly distributed throughout the lung pleura. The cervical lymph nodes were

Table 1 - Experimental collection schedule for fetal inoculation with *B. abortus* and condition at time of collection.

Fetus Number	Collection-date (day postinoculation)	Collection Status
Noninoculated Fetuses		
114	0	viable
560	0	viable
Saline Inoculated Fetuses		
54-110	4	viable
85-110-1	8	viable
85-110-2	8	viable
Brucella abortus Infected Fetuses		
148	1	viable
426	2	viable
163-1	3	viable
163-2	3	viable
161	3	dead (fresh)
526-1	4	viable
526-2	4	viable
143-1	6	dead (fresh)
143-2	6	dead (fresh)
243-1	6	dead (autolytic)
243-2	6	dead (autolytic)
666	6	born alive

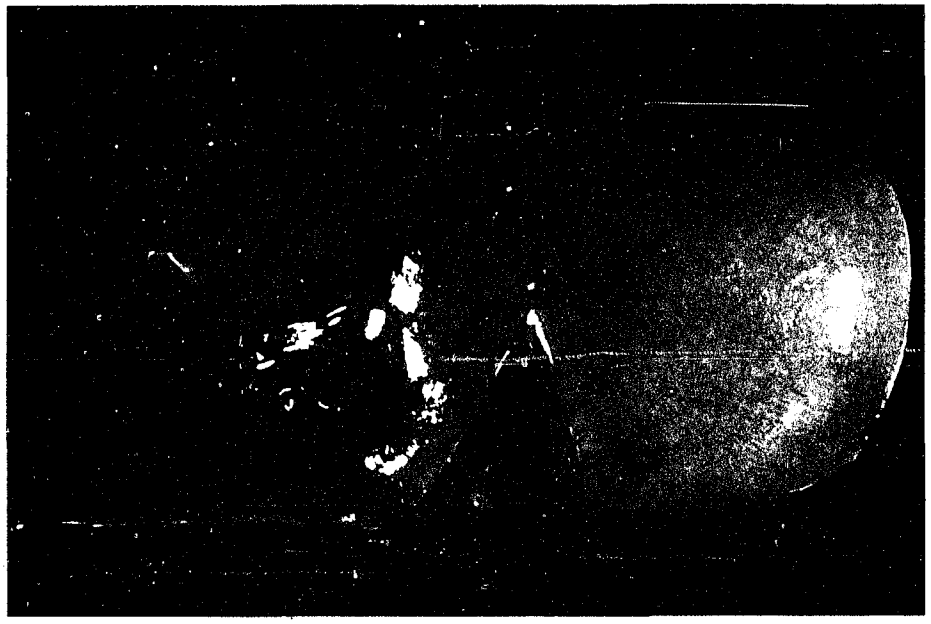

Fig 1 -- Lung from a saline inoculated fetus at postinoculation day 4.

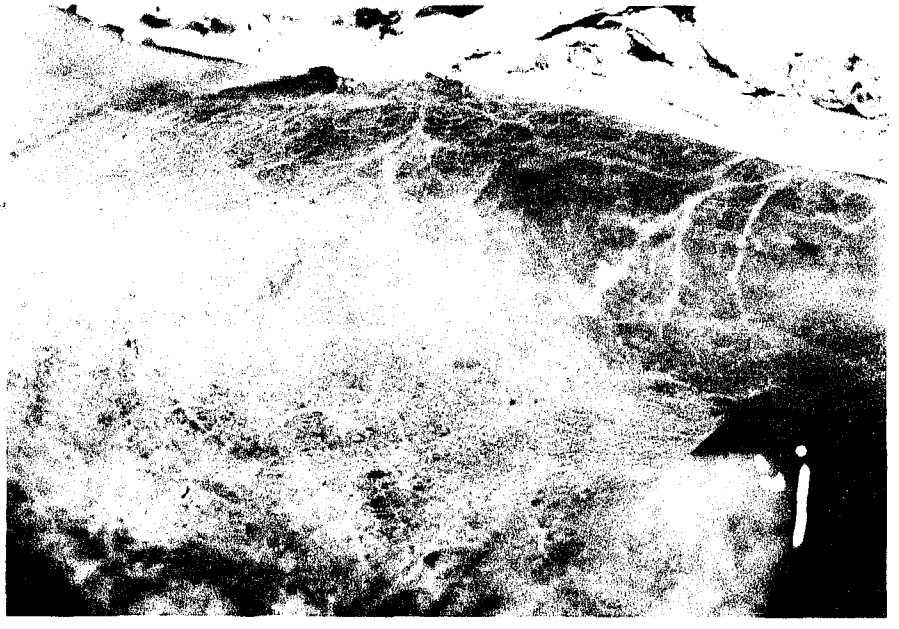

Fig 2 -- Lung from a B. abortus infected fetus at postinoculation day 3. Blood vessels are prominent and there is moderate perivascular edema. Petechial hemorrhagic areas are randomly distributed throughout the lungs.

slightly enlarged.

In the twin B. abortus infected fetuses at PI day 4, the cervical lymph nodes were moderately enlarged. In the lungs of fetus 526-2, there were multiple 1-2 mm, randomly distributed white foci. These foci extended into the lung parenchyma. Gross lesions were not present in the lungs of fetus 526-1.

In B. abortus infected fetuses at PI day 6 (143-1 and 143-2) the cervical lymph nodes were moderately enlarged and there were a variety of lung and abdominal organ changes. In these fetuses there was moderate to severe subcutaneous edema. The thoracic and abdominal cavities were filled with a red tinged watery fluid. Abdominal and thoracic organs were covered with a fibrillar, white material (fibrin) (Fig 3). There were fibrin adhesions between abdominal organs, abdominal organs and the abdominal wall and between the lung and the thoracic wall (Fig 4). In the other B. abortus infected fetus at PI day 6 (666), gross lesions were limited to the lungs and cervical lymph nodes. The lungs had several 3-5 cm dark red, firm areas. These areas were consolidated and sank in formalin. Fetuses 243-1 and 243-2 were extremely autolyzed. Macroscopic lesions were not discernible in these fetuses.

The lungs, lymph node, thymus, spleen, kidney, liver and adrenal glands of each fetus were evaluated microscopically. Significant lesions in B. abortus infected fetuses were present in the lungs and lymphoid organs.

Fig 3 -- Abdominal and thoracic fibrin covered organs from a B. abortus infected fetus at postinoculation day 6.

Fig 4 -- Fibrin adhesions between the liver and small intestine from a B. abortus infected fetus at postinoculation day 6.

In non-infected fetuses, the pulmonary alveolar walls were 1-3 cell layers thick (Fig 5). A few mononuclear and segmented leukocytes with large eosinophilic granules circumscribed a few interlobular blood vessels. Occasional mononuclear and segmented leukocytes with large eosinophilic granules were randomly distributed throughout the alveolar septal wall.

Cervical lymph nodes were slightly cellular. The cortices were thin with a few diffusely distributed lymphocytes and focal dense accumulations of lymphocytes. Medullary cords were populated by predominately lymphocytes, focal accumulations of mononuclear and segmented leukocytes with large eosinophilic granules, foci of neutrophils in different maturational stages and occasional extramedullary hematopoetic foci. Sinuses contained a few foamy macrophages, lymphocytes, occasional neutrophils and mononuclear and segmented leukocytes with large eosinophilic granules (Fig 6).

The thymic cortices were densely cellular and the medulla and trabecular connective tissue contained a few mononuclear and segmented leukocytes with large eosinophilic granules. In the spleen there were a few to moderate numbers of diffusely distributed extramedullary hematopoetic foci and central veins were circumscribed by a thin, moderately dense population of lymphocytes. Moderate numbers of diffusely distributed extramedullary hematopoetic foci were randomly distributed.

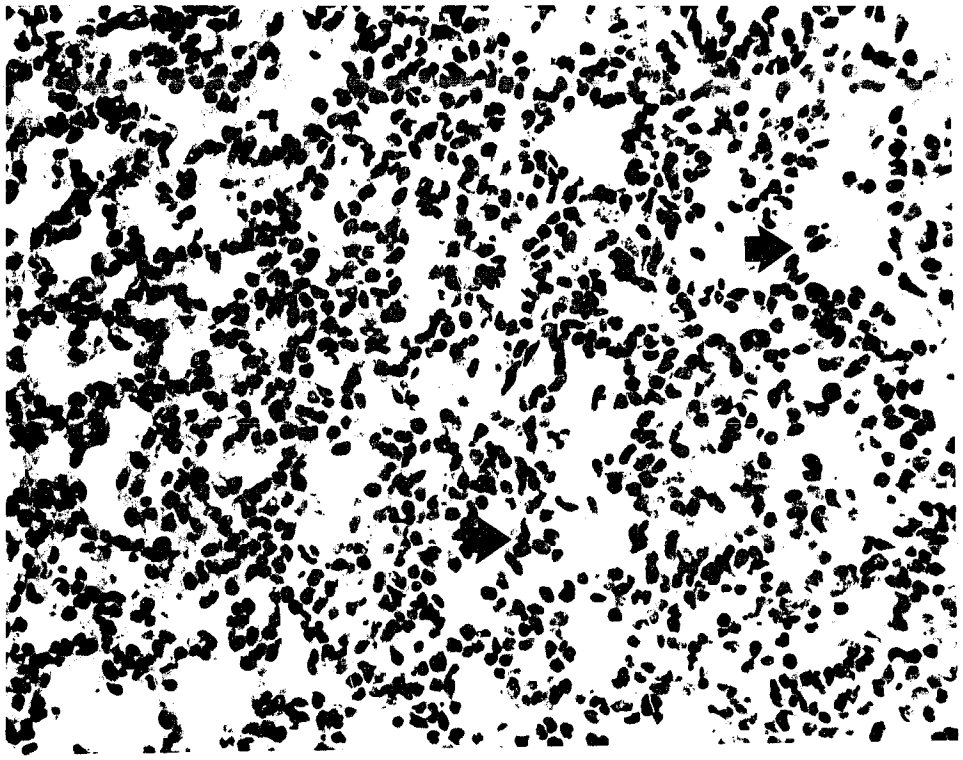

Fig 5 -- Lungs from a control fetus. Alveolar walls (arrows) are thin, 1-3 cell layers thick. H & E stains; X 400.

Fig 6 -- Lymph node from a control fetus. The sinuses (arrows) are sparsely populated by a few macrophages and neutrophils. H & E stain; X 160.

In the lungs of the B. abortus infected fetus at PI day 1, there was mild to moderate interlobular edema. Moderately increased numbers of interiobular perivascular spaces contained a few mononuclear and segmented globular leukocytes. Alveolar walls and alveolar blood vessels contained a few neutrophils and a few mononuclear and segmented leukocytes with large eosinophilic granules.

The thymic medulla contained moderately increased numbers of mononuclear and segmented leukocytes with large eosinophilic granules and interlobular trabeculae were slightly edematous. Focal interlobular leukocytic accumulations were comprised of moderate numbers of mononuclear and segmented leukocytes with large eosinophilic granules and a few band and mature neutrophils.

The small blood vessels in the kidney contained a few neutrophils. Microscopic lesions were not present in the spleen, liver, adrenal gland, and the cervical lymph node.

In lung peribronchiolar areas of the B. abortus infected fetus at PI day 2, alveolar walls were moderately thickened and interlobular trabeculae were moderately edematous with distended lymphatics and a few widely distributed neutrophils and segmented leukocytes with large eosinophilic granules. Alveolar wall thickening was principally due to large mononuclear cells with scant cytoplasm and vesicular nuclei (Fig 7). Occasional neutrophils and a small amount of granular eosinophilic material (meconium) were present in a few alveoli.

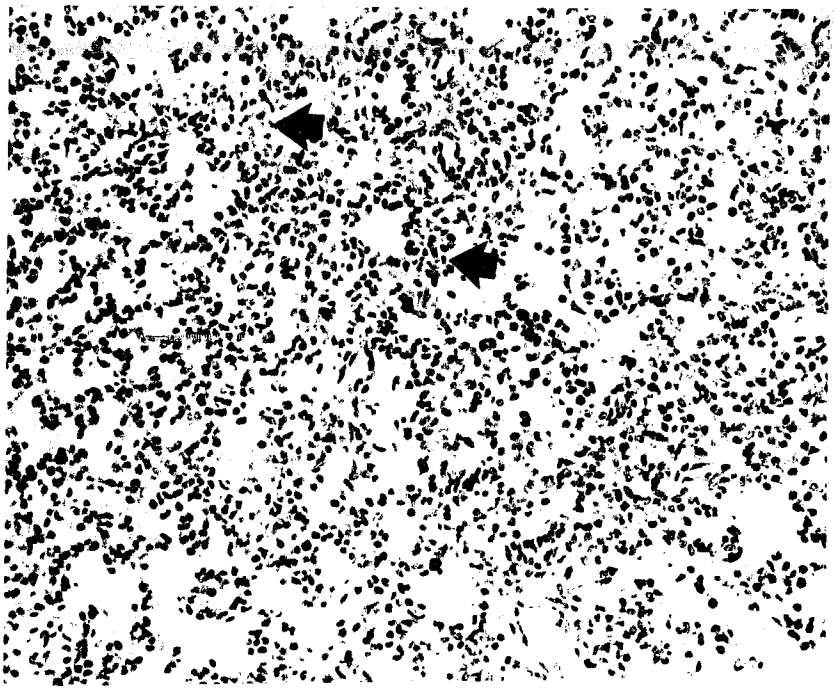

Fig 7 -- Lung from a B. abortus infected fetus at postinoculation day 2. Alveolar walls (arrows) are moderately thickened by large mononuclear cells. H & E stain; X 160.

In the cervical lymph node there was moderate increased cellularity. The cortex was moderately thickened by dense accumulations of lymphocytes and moderately cellular population of lymphocytes. Sinuses contained moderate numbers of large foamy macrophages. Moderately increased numbers of mononuclear and segmented globular leukocytes and mature neutrophils populated the medullary cords.

Histologic lesions in the thymus and the kidney were similar to histologic lesions in the thymus and kidney from the B. abortus infected fetus at PI day 1. In the spleen, there were dense accumulations of lymphocytes and moderate numbers of diffusely distributed neutrophils. No significant lesions were present in the liver and adrenal gland.

In B. abortus infected fetuses at PI day 3, microscopic lung lesions were similar to microscopic lung lesions in the B. abortus infected fetus at PI day 2.

The cervical lymph node histologic lesions were similar to but were more severe than histologic lesions in the PI day 2 cervical lymph node. In fetuses 85-116 and 85-163-1, the sinuses were filled with large macrophages with abundant eosinophilic cytoplasm (Fig 8). Many of these macrophages contain erythrocytes (erythrophagocytosis).

In thymic cortices, there was moderate decreased cellularity. Other thymic changes were similar to thymic changes in the PI day 1 fetus. Kidney and spleen changes were similar to changes in the B. abortus infected fetus at

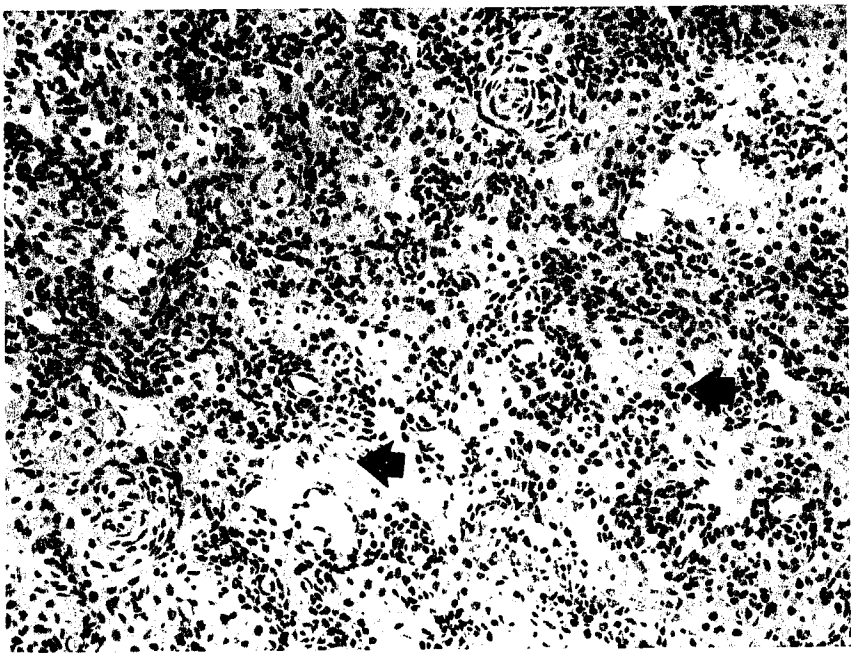

Fig 8 - Lymph node from a B. abortus infected fetus at postinoculation day 3. Medullary sinuses are filled with foamy macrophages (arrows). H & E stain; X 160.

PI day 2. No significant lesions were present in the liver and adrenal gland.

In the B. abortus infected fetus at PI day 4, fetus 526-1, histologic pulmonary lesions were similar to lung histologic lesions in the B. abortus infected fetus at PI day 2. Lung changes in 526-2 were characterized by peribronchiolar inflammation (Fig 9). In multifocal to confluent areas, peribronchiolar alveoli were filled with moderate numbers of neutrophils and a few mononuclear cells with large vesicular nuclei (Fig 10). Meconium was present in a few of these alveoli. Alveolar walls were moderately thickened by large mononuclear cells with large vesicular nuclei and scant cytoplasm.

Histologic lesions in the cervical lymph node, thymus, spleen and kidney were similar to histologic lesions in these organs in fetus 85-163, a B. abortus infected fetus at PI day 3. No significant lesions were present in the liver and adrenal gland.

In B. abortus infected fetuses at PI day 6, (fetus 143-1 and fetus 143-2), microscopic lesions were present in all organs examined except the liver. In the lungs, the pleura, interlobular septae and alveolar walls were moderately thickened and there were focally collapsed areas. The pleura and interlobular septae were diffusely edematous and congested with multiple hemorrhagic foci. A moderate amount of fibrin, a few macrophages and lymphocytes and a few neutrophils were diffusely distributed throughout the

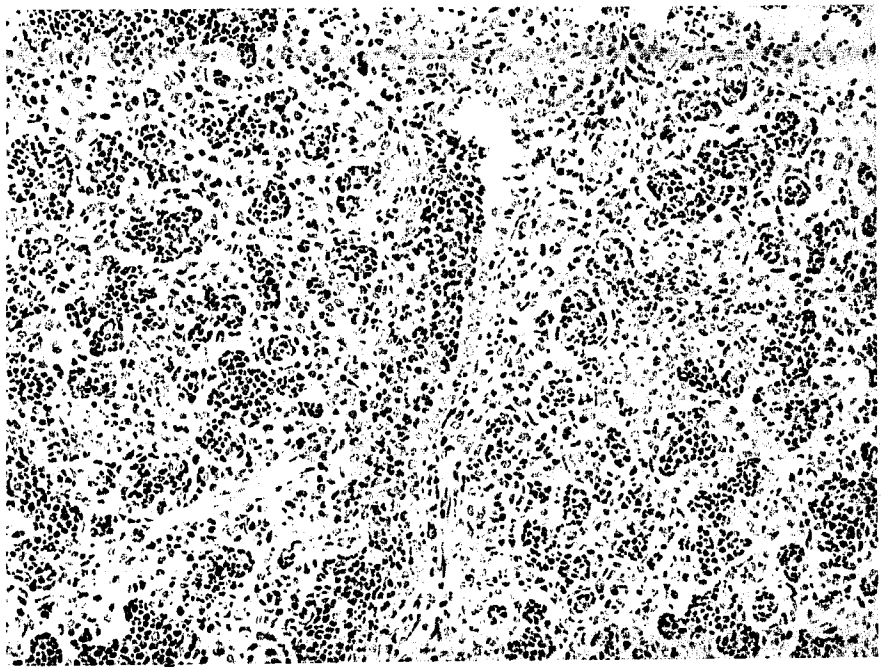

Fig 9 -- Lung from a B. abortus infected fetus at PI day 4. Bronchioles and alveoli are filled with neutrophils and macrophages. H & E stain; X 160.

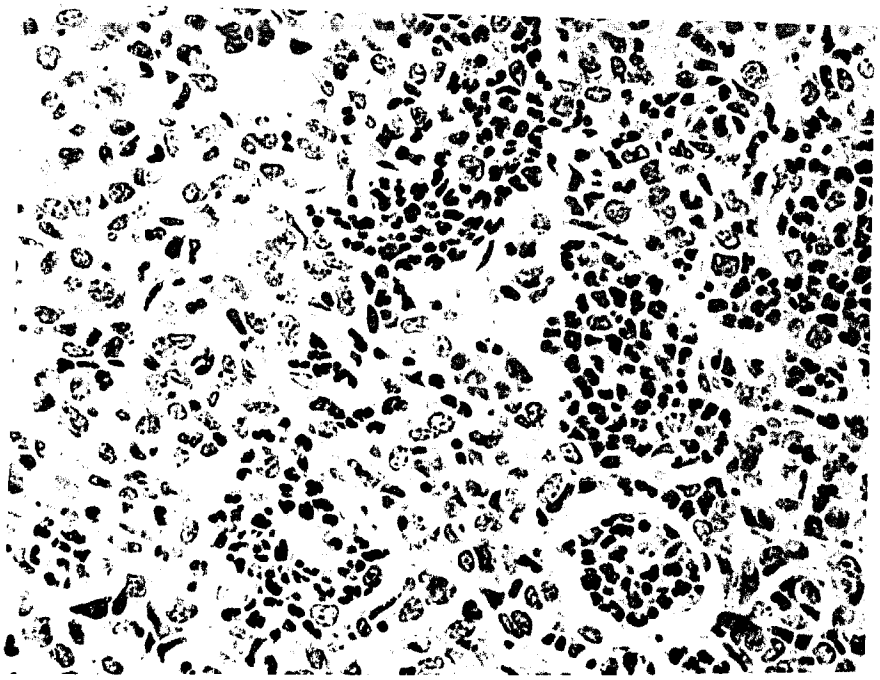

Fig 10 -- Lung from a B. abortus infected fetus at PI day 4. Alveoli contain large mononuclear cells surrounded by moderate numbers of neutrophils. H & E stain; X 400.

serosa and interlobular septae. Alveolar walls were 2-5 layers thick and alveoli were collapsed in a few areas. Alveolar wall thickening was due to large round cells with large vesicular nuclei, a few neutrophils and occasional mononuclear and segmented leukocytes with large eosinophilic granules. Meconium and a few neutrophils and macrophages were present in a few alveoli. Microscopic lung lesions of fetus 666 were characterized by a moderate to severe bronchopneumonia. Peribronchiolar alveoli were filled with neutrophils and macrophages, and a few contained meconium. In a few peribronchiolar areas, alveoli were collapsed.

Cervical lymph nodes were hypercellular. Cortices were moderately thickened by a dense population of lymphocytes. A few primary follicles were present in the lymph node from fetus 85-143-2 (Fig 11). Sinuses were filled with large foamy macrophages and a few lymphocytes. Predominately lymphocytes, moderate numbers of mononuclear, band and segmented leukocytes with large eosinophilic granules populated medullary cords.

The small intestinal serosa was markedly thickened by edema fluid, fibrin and a few lymphocytes and macrophages. Histologic lesions in the thymus, spleen, and kidney were similar to histologic lesions in these organs in fetus 85-163, a PI day 3 fetus. The adrenal gland was not evaluated histologically.

Because of advanced autolysis, tissues from B. abortus infected PI day 6 fetuses 243-1 and 243-2 were not processed

Fig 11 -- Lymph node from a B. abortus infected fetus at postinoculation day 6. There is a densely cellular cortex and a primary follicle (F). H & E stain; X 160.

for histologic evaluation.

Hematology -- The mean percentage of lymphocytes was markedly lower and the mean percentage of neutrophils was markedly higher in B. abortus infected fetuses than in uninfected fetuses (Table 2). A left shift of neutrophils with monocytosis was present in both B. abortus infected fetuses at PI day 4.

The absolute cell counts for neutrophils, band neutrophils, lymphocytes, monocytes and eosinophils were calculated (Table 3). The total number of leukocytes in the 5 viable uninfected control fetuses and 6 viable B. abortus infected fetuses were compared (Fig 12). In the B. abortus infected fetus at PI day 1, there was when compared with uninfected controls, a marked increased in total leukocyte numbers. The leukocyte totals from B. abortus infected fetuses at PI days 2, 3, and 4 gradually declined. In the B. abortus infected fetus at PI day 6 (fetus 666, blood sample collected 2 hours after birth), the total leukocyte count was markedly elevated.

In B. abortus infected fetuses, the total number of neutrophils were markedly elevated (Fig 13). The total number of lymphocytes (except in fetus 666) were consistently lower in B. abortus infected fetuses than in uninfected fetuses (Fig 14). Total monocyte numbers were similar in uninfected fetuses and B. abortus infected fetuses (Fig 15) except at PI day 4.

With the nonspecific esterase stain, monocytes stained

Table 2 - Blood leukograms from viable noninfected and *B. abortus* infected ovine fetuses.

Fetus Number	PI Days*	Leukocyte differential (percentage)					Total x10^3/ul
		L	S	B	M	E	
Uninfected controls							
56	0	83	11	0	4	2	4.4
114	0	82	14	1	4	3	2.4
54-110	4	87	10	0	1	2	2.7
85-110-1	8	83	12	0	3	2	2.7
85-110-2	8	83	15	0	2	0	2.6
B. abortus infected							
85-148	1	27	70	0	2	0	5.5
426	2	55	44	0	1	0	4.0
85-116	3	8	89	0	3	0	3.7
526-1	4	25	62	4	9	0	1.7
526-2	4	32	51	10	8	0	2.5
666	6	53	46	0	1	0	7.1

* PI Days = Time of fetus collection in days postinoculation

\# L = lymphocytes, S = mature neutrophils, B = band neutrophils, M = monocytes, E = eosinophils

Table 3 - Total blood leukocyte counts from viable noninfected and *B. abortus* infected ovine fetuses

Fetus Number	PI Days*	Leukocyte differential x10^3ul				
		L	S	B	M	E
Uninfected controls						
56	0	3.32	0.44	0.00	0.16	0.08
114	0	2.00	0.33	0.00	0.07	0.02
54-110	4	2.34	0.03	0.00	0.03	0.05
85-110-1	8	2.12	0.39	0.00	0.05	0.00
85-110-2	8	2.24	0.32	0.00	0.08	0.05
B. abortus infected						
85-148	1	1.50	3.85	0.00	0.11	0.00
426	2	1.76	2.20	0.00	0.04	0.00
85-116	3	0.29	3.30	0.00	0.11	0.00
526-1	4	0.43	1.05	0.07	0.15	0.00
526-2	4	0.80	1.27	0.25	0.20	0.00
666	6	3.05	3.76	0.00	0.29	0.00

* PI Days = Time of blood collection in days postinoculation

\# L = lymphocytes, S = mature neutrophils, B = band neutrophils, M = monocytes, E = eosinophils

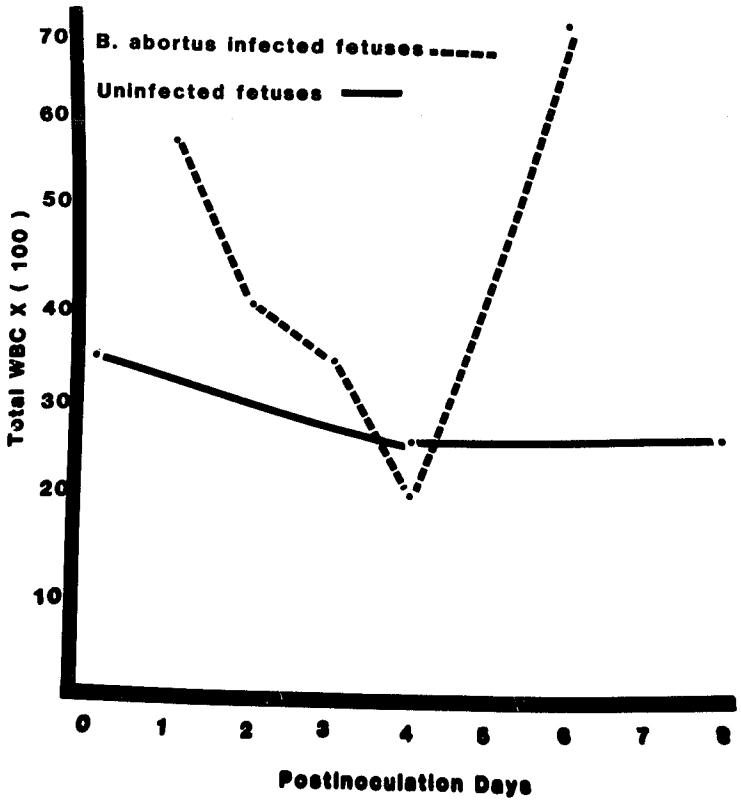

Fig 12 -- A comparison of total leukocyte counts from uninfected control and B. abortus infected fetuses.

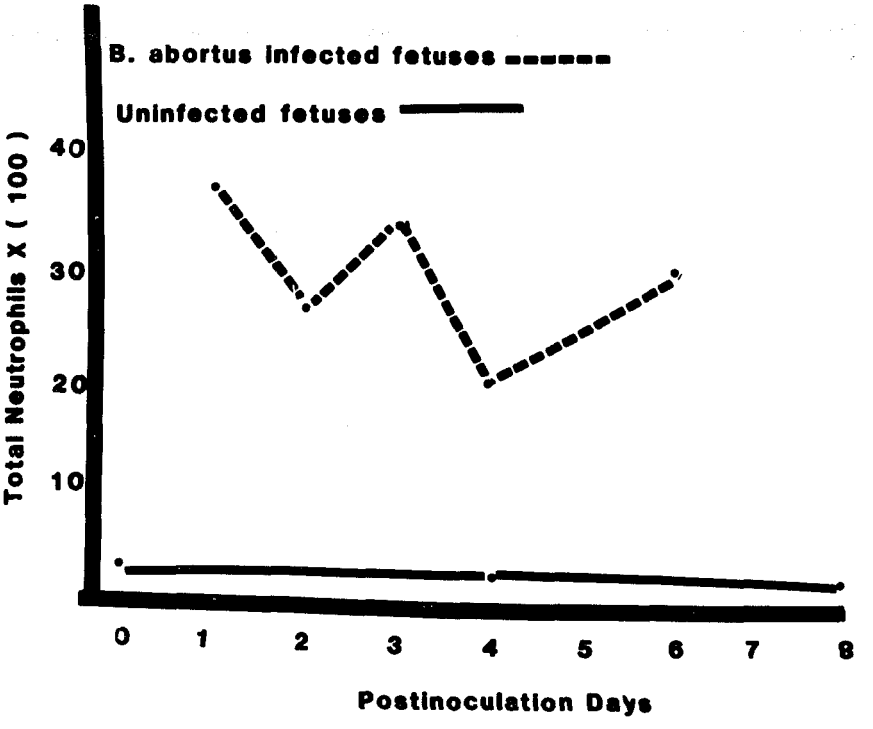

Fig 13 -- A comparison of total number of neutrophils from uninfected control and B. abortus infected fetuses.

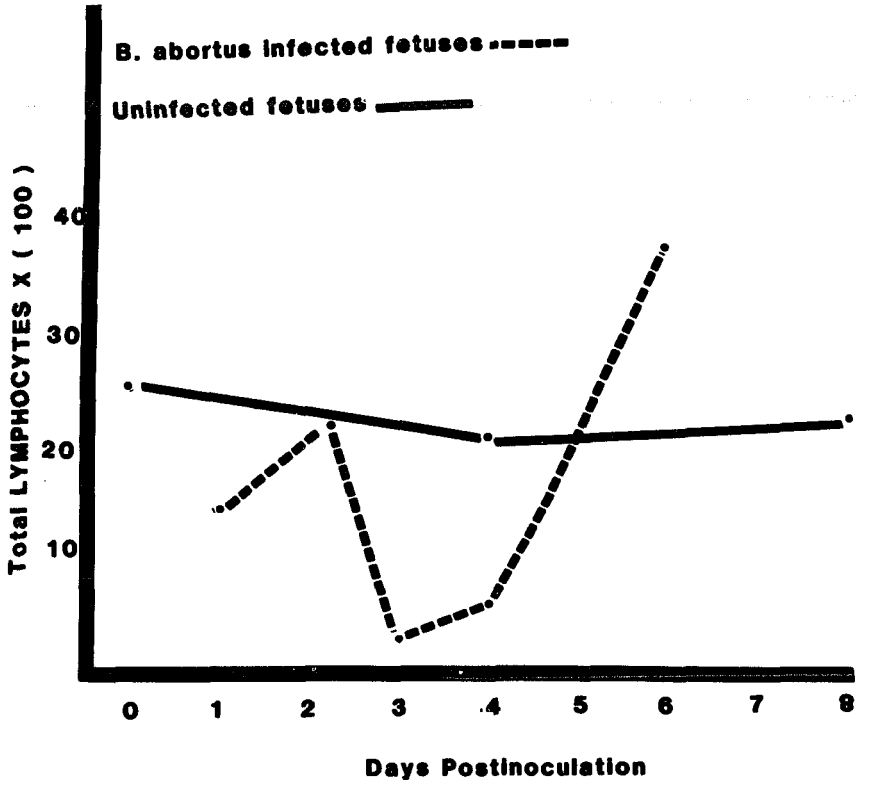

Fig 14 -- A comparison of total lymphocyte counts in uninfected control and B. abortus infected fetuses.

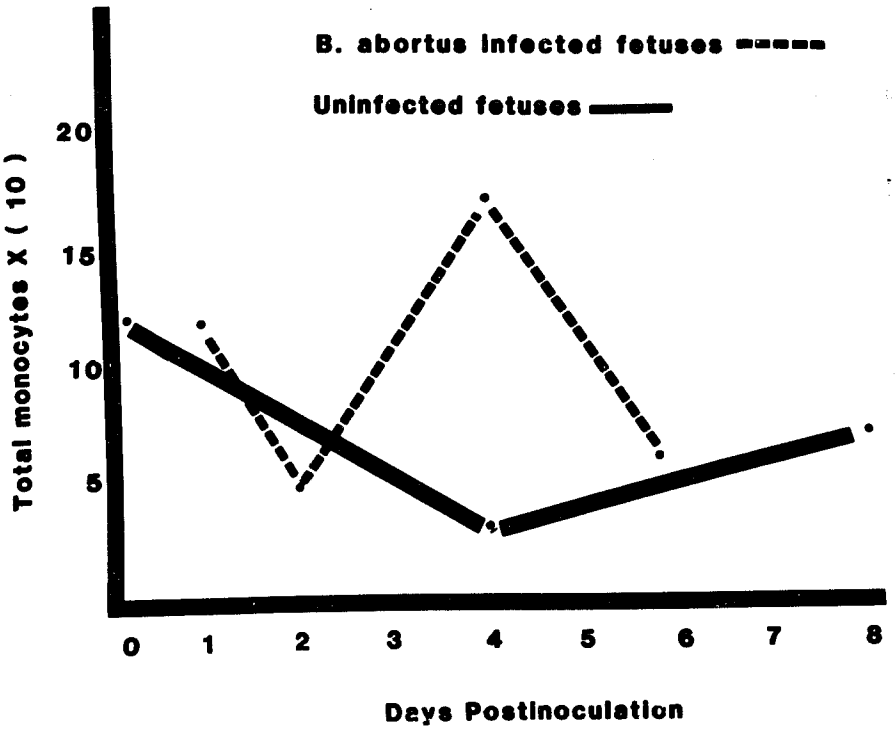

Fig 15 -- A comparison of total number of monocytes from uninfected control and B. abortus infected fetuses.

bright red. Leukocyte differential counts obtained using the nonspecific esterase stain were similar to those obtained using the modified Wright's stain.

Neutrophil Functions -- Phagocytosis of B. abortus and S. aureus by neutrophils from uninfected control fetuses and B. abortus infected fetuses in autologous serum, fetal calf serum and Gey's solution was determined (Table 4). In uninfected control fetuses, there were no major differences in the percentage of neutrophils that phagocytosed B. abortus or S. aureus in autologous serum, fetal calf serum or Gey's solution.

The percentage of neutrophils from uninfected controls fetuses that phagocytosed B. abortus and S. aureus in autologous serum was compared to the percentage of neutrophils from B. abortus infected fetuses that phagocytosed B. abortus (Fig. 16) and S. aureus (Fig. 17) in autologous serum. The percentage of neutrophils from B. abortus infected fetuses that phagocytosed B. abortus and S. aureus in autologous serum was markedly elevated when compared to the percentage of neutrophils from uninfected control fetuses that phagocytosed B. abortus and S. aureus.

In B. abortus infected fetuses, the percentage of neutrophils that phagocytosed S. aureus in autologous serum was markedly greater than the percentage of neutrophils that phagocytosed B. abortus in autologous serum before PI day 4. At PI days 4 and 6 there was no discernible differences in the phagocytosis of S. aureus and B. abortus in autologous

Table 4 - The phagocytosis of *B. abortus* and *S. aureus* by neutrophils from *B. abortus* infected and uninfected fetal sheep

PI Days Uninfected controls	(n)	Phagocytosis Percentage BA	BF	BG	SA	SF	SG
0	(2)	32	13	15	35	23	24
4	(1)	16	0	0	33	23	19
8	(1)	3	0	0	18	31	5
B. abortus infected							
1	(1)	2	0	0	65	42	4
2	(1)	45	2	0	85	58	3
3	(1)	6	3	0	78	41	18
4	(2)	80	48	17	94	91	91
6	(1)	74	0	0	78	53	59

PI Days = Days postinoculation, n = number of fetuses, B = *B. abortus*, A = autologous serum, F = fetal calf serum, G = Gey's solution, S = *S. aureus*.

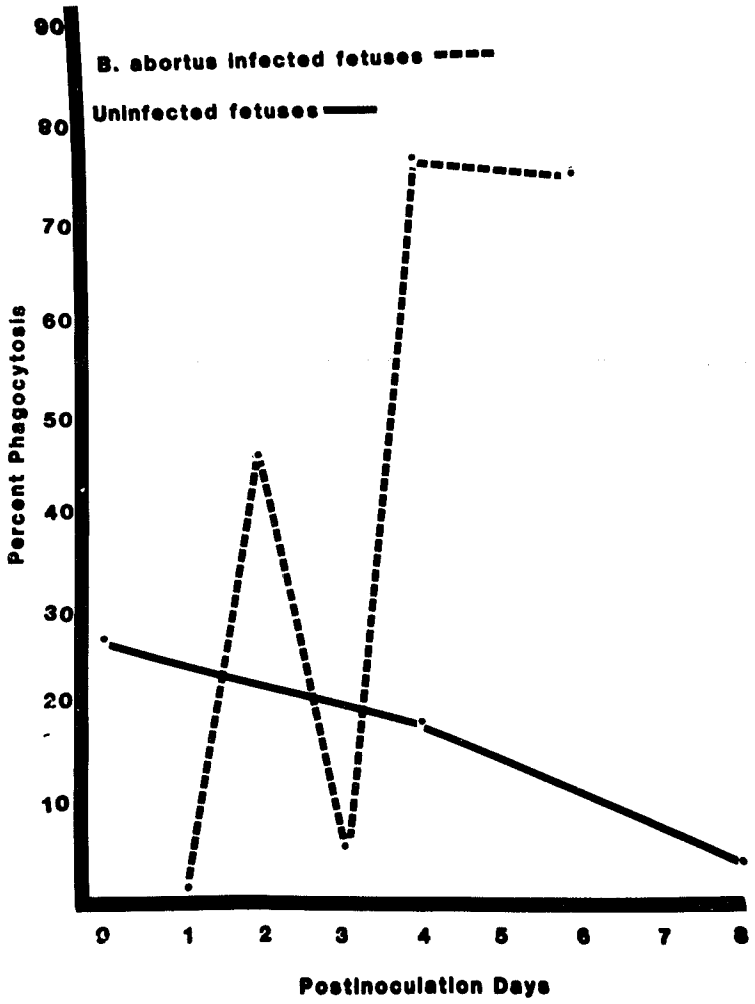

Fig 16 -- A comparison of the percent of neutrophils from uninfected control fetuses and B. abortus infected fetuses that phagocytosed B. abortus in autologous serum.

Fig 17 -- A comparison of the percent of neutrophils from uninfected control fetuses and B. abortus infected fetuses that phagocytosed S. aureus in autologous serum.

serum.

Major differences were not observed in the percentage of neutrophils from uninfected fetuses that phagocytosed B. abortus and S. aureus in fetal calf serum and Gey's solution, and the percentage of neutrophils from B. abortus infected fetuses that phagocytosed B. abortus and S. aureus in fetal calf serum and Gey's solution.

There were low levels of killing S. aureus and B. abortus by neutrophils from all uninfected control fetuses and most B. abortus infected fetuses in autologous serum, fetal calf serum and Gey's solution (Table 5).

Serology -- Using the ELISA test, all serum samples from uninfected control fetuses and B. abortus infected fetuses were negative for antibodies to B. abortus. With the Mancini technique, trace levels of either IgM, IgG_1 or IgG_2 immunoglobulin were detected in B. abortus infected fetuses at PI days 2, 4, and 6. Serum samples from uninfected control fetuses and B. abortus infected fetuses at PI days 1 and 3 (85-148 and 85-161) were negative for IgM, IgG_1 and IgG_2 antibodies.

All serum samples from uninfected control fetuses and from B. abortus infected fetuses were card test and complement fixation test negative for antibodies to B. abortus.

Cortisol Levels -- Cortisol levels from uninfected control fetuses and B. abortus infected fetuses were compared (Fig. 18). Total cortisol levels of B. abortus

Table 5 - The killing of *B. abortus* and *S. aureus* by neutrophils from *B. abortus* infected and uninfected fetal sheep

PI Days Uninfected controls	(n)	Killing Percentage BA	BF	BG	SA	SF	SG
0	(2)	22	0	0	8	0	0
4	(1)	8	0	0	28	39	33
8	(1)	0	0	0	0	0	0
B. abortus infected							
1	(1)	33	0	0	16	2	0
2	(1)	68	0	0	24	11	50
3	(1)	100	33	0	53	48	25
4	(2)	32	17	0	9	8	2
6	(1)	12	0	0	71	38	0

PI Days = Days postinoculation, n = number of fetuses, B = *B. abortus*, A = autologous serum, F = fetal calf serum, G = Gey's solution, S = *S. aureus*.

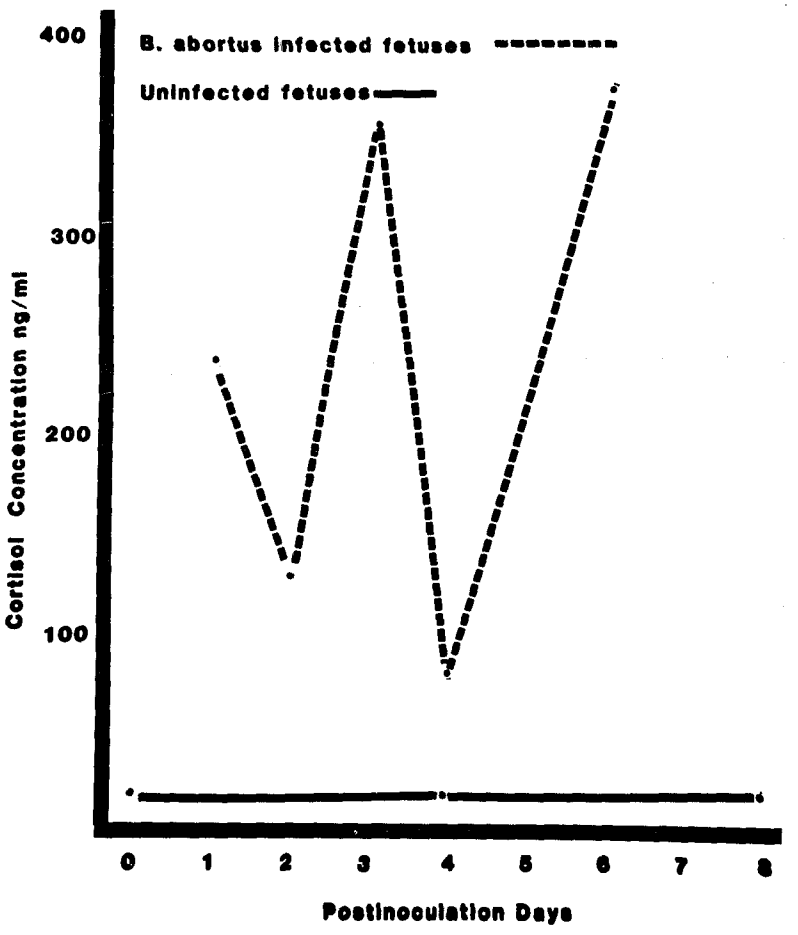

Fig 18 -- A comparison of serum cortisol levels in uninfected control fetuses and B. abortus infected fetuses.

infected fetuses were markedly higher (3-36 times greater) than total cortisol levels in uninfected fetuses. There was no relationship between cortisol elevations and duration of infection.

Discussion

Brucella abortus was isolated from all cultured tissues collected from *B. abortus* infected fetuses. The isolation of *B. abortus* from the *B. abortus* infected fetus at PI day 1 was evidence that a bacteremia developed within 24 hours of fetal infection. Isolation of *B. abortus* from severely autolyzed *B. abortus* infected twin fetuses at PI day 6 was evidence that *B. abortus* could be isolated from decomposed ovine fetuses. The high recovery rate of *B. abortus* from infected fetuses in this study is consistent with previous findings.[31,32] Shaw[32] recovered *B. abortus* in 5 of 5 naturally infected aborted ovine fetuses and Luchsinger[31] cultured *B. abortus* from 4 of 4 naturally infected, aborted ovine fetuses.

The gross lesions in *B. abortus* infected ovine fetuses occurred sooner and were more severe than reported in *B. abortus* infected bovine fetuses.[1-4,29] Vascular changes appeared to be more prominent in infected fetal sheep than in infected fetal cattle. The fibrin covered thoracic and abdominal organs observed in infected ovine fetuses in this study were more extensively involved than has been reported

in infected bovine fetuses. In B. abortus infected ovine fetuses at PI day 6, subcutaneous edema, multiple variable sized, white lung foci and a red tinged thoracic and abdominal fluid were similar to lesions in B. abortus infected bovine fetuses.[1,4,29]

Microscopic lesions in B. abortus infected ovine fetuses were similar to those reported microscopic changes in B. abortus infected bovine fetuses.[1-4,29] Lymphatic and vascular alterations and edema and fibrin production appeared to be more prominent in ovine fetuses. These findings suggest that fetal lambs may be more susceptible to B. abortus than fetal cattle although differences in the dose and route of inoculation may have influenced the pathogenesis.

Typical bovine fetal brucella bronchopneumonia has been reported as more severe and extensive, microscopically, than the ovine fetal bronchopneumonia observed in this study. The severe bovine fetal bronchopneumonia may be a consequence of time as most B. abortus infected bovine fetuses were examined after abortion, thus there was more time for lesion development. The fact that no B. abortus infected ovine fetus collected via ceasarean section at PI day 6 was alive, and the lamb born prematurely was weak at birth suggested that B. abortus was more pathogenic for ovine fetuses than bovine fetuses.

A consistent microscopic lung alteration in B. abortus infected fetuses was slightly to moderately thickened

alveolar walls. The alveolar wall thickeneing was due primarily to large mononuclear cells with scant cytoplasm. It was not possible to determine if these cells were macrophages or type II pneumonocytes.

If type II pneumocytes were the cells in alveolar walls, their proliferation was possibly induced by high serum concentrations of cortisol in the B. abortus infected ovine fetuses. Type II pneumocytes in fetuses were stimulated by glucocorticosteroid inoculation of pregnant dams or fetuses.[88,89]

Lymph node reactions to B. abortus infection were evident as early as PI day 3 when sinuses were filled with large foamy macrophages. The presence of primary lymphoid follicles in a B. abortus infected fetus at PI day 6 was evidence that immunological stimulation had occured, possibly due to B. abortus infection.

The presence of mononuclear, band and segmented leukocytes with large eosinophilic granules in the lymph node and thymus of B. abortus infected fetuses suggest that these cells were actively responding to B. abortus infection. These granulocytic cells were metachromatic when stained with new methylene blue therefore, they were possibly eosinophils, mast cells or basophils. However, the presence of foci of cells with mononuclear, band and segmented nuclei may have been produced locally. Whether these cells were locally produced or migrated into the tissues was not determined.

The increased total leukocyte counts, increased total neutrophil counts and decreased total lymphocyte counts in B. abortus infected fetuses were probably resultant of stress of bacterial infection. Stress was evident by the extremely high serum cortisol concentrations in B. abortus infected fetuses. The mature and immature neutrophil elevation and the monocytosis in B. abortus infected fetuses at PI day 4 were evidence of a hematologic response to B. abortus infection. The hematological response at PI day 4 indicated that fetuses possess the ability to elicit an active hematological response to B. abortus infection. Bacterial endotoxins possibly contributed to the increased total leukocyte count by causing the release of mature and immature neutrophils from the marrow pool as occurs in adults.[90-92]

The increase in the percentage of neutrophils from B. abortus infected fetuses that phagocytosed B. abortus in autologous serum was possibly due to increased levels of immunoglobulins and opsonization which allowed greater phagocytosis or possibly undefined serum factors. Fetuses 85-148 and 85-116 (B. abortus infected fetuses at PI days 1 and 3) were negative for immunoglobulins and the percentage of neutrophils from these B. abortus infected fetuses that phagocytosed B. abortus was markedly lower than the percentage of neutrophils that phagocytosed B. abortus from other B. abortus infected fetuses that contained trace levels of immunoglobulins.

Increased phagocytosis of S. aureus by neutrophils from B. abortus infected fetuses did not correlate with the appearance of trace levels of immunoglobulins. In autologous serum, a marked increase in phagocytosis of S. aureus occured at PI day 1 and persisted throughout this study. Possibly undefined serum factors were responsible for this rapid rise in phagocytosis of S. aureus.

Neutrophil killing of B. abortus and S. aureus was not enhanced or inhibited by B. abortus infection.

Cortisol serum concentrations increased drastically after B. abortus infection and remained elevated throughout this study. Elevated cortisol levels have been reported to induce premature parturition in fetal sheep.[80] High serum cortisol levels in these fetal sheep may have been responsible, at least in part, for the premature termination of pregnancy in some (6 or 12) B. abortus infected fetuses.

In conclusion, the fetal lamb is acceptable as an experimental model for fetal bovine brucellosis. This is supported by the similarities in morphologic lesions between B. abortus infected fetal sheep and cattle and in utero deaths of B. abortus infected fetal sheep and cattle. Additionally, B. abortus infection in ovine fetuses enhanced neutrophil phagocytic abilities, induced an active hematological response and elevated serum cortisol levels.

Chapter III

Morphologic Lesions, Hematological and Immunological Responses and Neutrophil Functions in Neonatal Sheep Infected with *Brucella abortus*

Introduction

Neonatal animals are highly susceptible to infectious diseases. This susceptibility is probably not a consequence of phagocyte dysfunction, as phagocytes from neonates function as efficiently as those in adults.[65,93] Inadequate hematological responses can be effectively eliminated as a significant predisposing factor to neonatal disease susceptibility because neonatal hematological responses to infectious organisms are comparable to adult hematological responses.[63] Schwartz reported that the degree of necrosis and cellular components were similar in neonatal and yearling monkeys inoculated subcutaneously with turpentine. Hypogammaglobulinemia and agammaglobulinemia frequently predispose neonates to morbidity and mortality due to infectious agents.[93-95] This finding is supported by the observation that calves with low blood immunoglobulin levels often develop gastrointestinal tract infections and die.[93-95] Additionally, nonheat-inactivated serum from normal newborn calves lacked the ability to kill *Staphylococcus aureus* or *Escherichia coli*. Nonheat-inactivated serum from adult cattle was

bactericidal to both organisms.[65]

Neonatal calves are extremely resistant to Brucella abortus infection. Most calves infected before 9 months of age rid themselves of the infection prior to adulthood.[1-3,12] A few animals infected as calves may become serologic positive to B. abortus and shed the organism immediately prior or after abortion or parturition (latently infected animals).[23,24] Factors responsible for neonatal resistance to B. abortus infections are undetermined or poorly understood.

In this study the neonatal sheep was evaluated as a model for bovine brucellosis. The factors that may explain neonatal resistence to B. abortus infection were examined. These studies included examination of histologic lesions, immunological and hematological responses and neutrophil functions in neonatal sheep infected with B. abortus Strain 2308.

Materials and Methods

Experimental Design -- Four 2-week-old lambs were infected with B. abortus and 3 2-week-old lambs were inoculated with physiologic saline. Blood and serum samples were collected before inoculation and at postinoculation (PI) days 2, 4, 8 and 16. All lambs were sacrificed at PI day 17.

Animals -- A total of 7 1-week-old crossbred lambs (2

males and 5 females) were obtained from the Louisiana State University sheep farm. Animals were housed in an enclosed barn, 4 and 3 per stall. They were fed lamb milk replacer, protein pellets and tap water. All animals were negative for antibodies to B. abortus by the card test (buffered brucella antigen) and complement fixation test.

Three lambs were used as saline inoculated controls and 4 lambs were inoculated with B. abortus Strain 2308. Inoculation was made in the dorsal neck muscles at 2 weeks of age. Blood was collected before inoculation and at PI days 2, 4, 8, and 16 days in dipotassium ethylenediaminotetraacetate (EDTA) and siliconized glass tubes. Blood collected in EDTA was used in neutrophil function tests, to obtain total leukocyte counts and to make blood smears. Serum from blood collected in siliconized tubes was used in brucella serology tests and as autologous serum in neutrophil function tests. Selected tissues were collected for culture and for histologic evaluation. Tissues collected for histologic evaluation were fixed in 10 percent formalin at 4° C.

Culture Procedures -- Tissues including lung, kidney, spleen, liver, intestinal lymph node, and cervical lymph node were collected using aseptic technique for bacteriological analysis. Tissues were cultured immediately or stored overnight at 4°C and cultured within 24 hours of harvest.

Tissues were prepared for culture by cutting them into

2-3 mm pieces with a sterile surgical blade. Tissue were placed in a sterile blender with 10 to 30 milliliters (ml) of sterile saline. The tissue saline mixture was blended for 20-30 seconds. Minced tissues were streaked on Tryptic Soy agar plates[a] with 5 percent heat-inactivated horse serum and brucella selective media[81] (with the addition of 0.2 g L cysteine, 1:5000 and 0.25 g erythritol, 1:4000 per liter). All plates were incubated for 2-5 days at 37°C.

B. abortus was identified as small round, pale-honey colored colonies. These bacteria were coccobacilli, Gram stain negative, urease and oxidase positive.

Bacteria -- Stock cultures of Staphylococcus aureus strain 520A[b] were stored at 4°C on Tryptose Soy agar. For daily use, stock S. aureus was inoculated in 5-10 ml of Tryptose broth and incubated 16-20 hours at 37°C. Bacteria were washed 3 times in physiologic Gey's solution and centrifuged 1600 x g for 10 minutes at 4°C. The pellet was suspended in 5 ml of physiologic Gey's solution. The absorbance reading was adjusted to .20 - .25 optical density (OD).[c] Bacteria were cultured on Tryptose Soy agar plates for determination of the number of bacteria per ml. The microdrop plating method was used for this determination.

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[a]Difco Laboratories, Detroit, Michigan.
[b]Dr. H. Cox, Department of Microbiology and Parasitology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803.
[c]Spectronic 20 Spectrophotometers, Bausch and Lomb, Inc. Rochester, New York.

B. abortus Strain 2308[d] stock was maintained in sterile saline. Stock B. abortus was inoculated in Tryptic broth and incubated at 37° C for 16-20 hours. Bacteria were washed 3 times in physiologic Gey's solution and centrifuged 1600 x g for 10 minutes at 4° C and the pellet was suspended in 5 ml of physiologic Gey's solution. The absorbance level was adjusted to .02 - .04 OD. Bacteria were cultured on Tryptic Soy agar plates enriched with 5 percent heat-inactivated horse serum for determination of the number of bacteria per ml. The microdrop plating method was used for this determination.

Tissue Processing -- Freshly collected sections of liver, lung, spleen, kidney, intestinal lymph node and cervical lymph node were fixed in 10 percent formalin at 4° C. Tissues were stored in cold formalin. Tissues were embedded in plastic as previously described.[82] A 5 g pack of white powder, benzoyl peroxide (catalyst), was added to 5 g of a liquid, 2-butoxy ethanol (solution B), and mixed with a magnetic stirrer. Infiltrating solution was prepared by mixing 5 ml of solution B with 50 ml of hydroxy ethyl methacrylate (solution A). Sections of tissues were added to this infiltrating solution and placed in a vacuum dessicator. A vacuum was established and maintained at 4°C for 24 hours. After 24 hours the infiltrating solution was

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[d]Dr. B. L. Deyoe, National Animal Disease Center, Agriculture Research Service, United States Department of Agriculture, Ames, Iowa 50010

changed and another vacuum was created and maintained at 4°C for an additional 24 hours.

The embedding solution was prepared by mixing 50 ml of solution A with 5 ml of solution B and 2 ml of N-N-dimethyl aniline (solution C). Two to three ml aliquots were pipetted into the lower depressions of plastic molds. Infiltrated tissue specimens were placed in the molds and covered with the embedding solution. The molds were placed in a vacuum dessicator. A vacuum was created and the vacuum dessicator was maintained at 4°C for 48 hours to allow polymerization.

Using a microtome[e] with a glass knife holder, plastic embedded tissues were cut in 2 micron sections with a glass knife. Sections were stained with hematoxylin and eosin. Selected tissues were stained with nonspecific esterase.

Hematology -- Blood was collected in EDTA tubes. This blood was used for preparation of blood smears. Total leukocyte counts were obtained with an electronic cell counter.[f] Blood smears were stained with a modified Wright's stain.[g] All blood smears were examined with an oil immersion lens for differential cell counts. Additionally, blood smears were stained for nonspecific esterase after fixation for 60 seconds in a pH 6.16 fixative

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[e]Sorvall Porter-Blum Microtome, Ivan Sorvall Incorporated, Newtown, Connecticut 06470.
[f]Houlter Electronics Incorporated, Hialeah, Florida.
[g]Diff Quik, American Scientific Products, Division of American Hospital Supply Corporation, McGraw Park, Il.

composed of KH_2PO_4, 100 mg; distilled H_2O, 30 ml; acetone, 45 ml; and formalin (30%), 25 ml.[83] Nonspecific esterase staining was obtained by incubating fixed blood smears in a solution composed of: Sorenson's phosphate buffer, 44.5 ml; hexazotized pararosaniline, 0.25 ml; and alpha-naphthyl butyrate solution, 3ml. Incubation was for 45 minutes in a 37°C water bath. Smears were washed in distilled water and counter stained in a 0.5 percent methyl green solution for 1 minute. Smears were then washed 3 times in distilled water. They were air dried and coverslipped. All smears were examined with an oil immersion lens for differential cell counts and nonspecific esterase positive (monocytes) and negative cells.

Neutrophil Phagocytosis and Killing Assay -- Neutrophil phagocytosis and killing functions were assayed by modifications of Pantazis' method[84] using acridine orange.[h] Blood collected in EDTA was washed 2 times in physiologic saline and once in physiologic Gey's solution followed by centrifugation at 400 x g for 10 minutes at 4°C. Approximately 0.5 x 10^6 leukocytes (in 0.3 ml of pelleted blood) were mixed with 30 x 10^6 bacteria (S. aureus Strain 520A or B. abortus Strain 2308 suspended in 0.5 ml of physiologic Gey's solution) and 0.2 ml serum (autologous or fetal calf serum) or 0.2 ml. physiologic Gey's solution. Six assay mixtures prepared on each sample included:

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[h]Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, New Jersey, 07410.

pelleted blood and B. abortus mixed with 1) autologous serum, 2) fetal calf serum, and 3) physiologic Gey's solution, and pelleted blood and S. aureus mixed with, 4) autologous serum, 5) fetal calf serum, and 6) physiologic Gey's solution. The 6 samples were incubated for 60 minutes in a 37°C water bath that rotated 200 revolutions per minute. After incubation, all tubes were vortexed and centrifuged at 400 x g for 10 minutes at 4°C. All samples were stored at 4°C and examined within 4 hours.

The supernatant was removed and a drop of pelleted blood and a drop of 0.14 percent acridine orange were added to a clean glass slide and coverslipped. Cells were examined with a fluorescent microscope.[i] The 63X and the 100X oil immersion lens and an epifluorescent light source with a 510mm filter were used to examine wet mounts. Three replicates of 100 leukocytes were counted for each assay mixture. Neutrophils that ingested 1 or more bacterial organisms were considered phagocytic. Neutrophils that contained 1 or more dead (red) bacterial organisms were counted as neutrophils which had killed bacteria.

Calculations of phagocytic and killing functions were obtained using the formulas:

$$\text{Phagocytosis \%} = \frac{\text{neutrophils ingesting 1 or more organism}}{\text{total neutrophils counted}} \times 100$$

[i]Carl Zeiss, D 7082 Oberkochen, West Germany.

$$\text{Killing \%} = \frac{\text{neutrophils containing 1 or more dead organisms}}{\text{total neutrophils ingesting bacteria}} \times 100$$

Baseline phagocytic and killing levels were obtained in day 0 lambs (before saline or B. abortus inoculation). The change in neutrophils that phagocytosed and killed B. abortus and S. aureus after B. abortus infection was compared with the change in neutrophils that phagocytosed and killed B. abortus and S. aureus after saline inoculation.

Serology -- The card (buffered brucella antigen) and the complement fixation test were used to detect brucella specific antibodies.[14,61]

Statistical Analysis -- Data were evaluated by analysis of variance with the Duncan's Multiple Range for means separation.

Results

Culture -- All uninfected lambs were negative for B. abortus and all B. abortus infected lambs were positive for B. abortus from 2-5 organs (Table 1).

Pathology -- Gross abnormalities were limited to the cervical lymph nodes. The nodes from lambs 2, 3 and 4 (3 of 4 B. abortus infected lambs) were moderately (2 times normal size) and uniformly enlarged.

Table 1 - B. abortus recovered from tissues of B. abortus infected lambs.

Cultured Tissues	Lamb 1	Lamb 2	Lamb 3	Lamb 4
Lung	-	-	+	+
Liver	+	+	+	-
Kidney	+	-	+	+
Spleen	+	-	+	+
Cervical Lymph Node	+	+	-	+
Intestinal Lymph Node	-	-	+	-

B. abortus culture results.

Cervical lymph nodes from uninfected control lambs were characterized by moderately thick cortices and moderate diffuse cellularity. Present in the cortices were a few primary follicles, a dense population of lymphocytes, focal accumulations of neutrophils and a few widely distributed segmented leukocytes with large eosinophilic granules. Medullary cords were comprised of moderate numbers of lymphocytes, a few eosinophils, neutrophils and occasional plasma cells. Sinuses contained a few foamy macrophages and a few lymphocytes.

Histologic changes in cervical lymph nodes from B. abortus infected lambs varied from moderate cortical depletion and a few primary follicles to markedly thickened cortices with numerous prominent lymphoid follicles and diffuse dense cellularity. In Lamb 3, the cellularity of the cortex and the medullary trabeculae was moderately decreased. The cellular component of the medullary trabeculae was comprised of lymphocytes, plasma cells and occasional eosinophils and neutrophils. All sinuses were prominent and filled large macrophages with abundant eosinophilic cytoplasm (Fig 1). The cortices of cervical lymph nodes from lambs 1, 2 and 4 were markedly thickened by numerous prominent primary lymphoid follicles and a dense population of lymphocytes (Fig 2). Medullary cords contained lymphocytes, plasma cells, few to moderate numbers of mononuclear, band and segmented leukocytes with large eosinophilic granules and a few neutrophils. Sinuses were

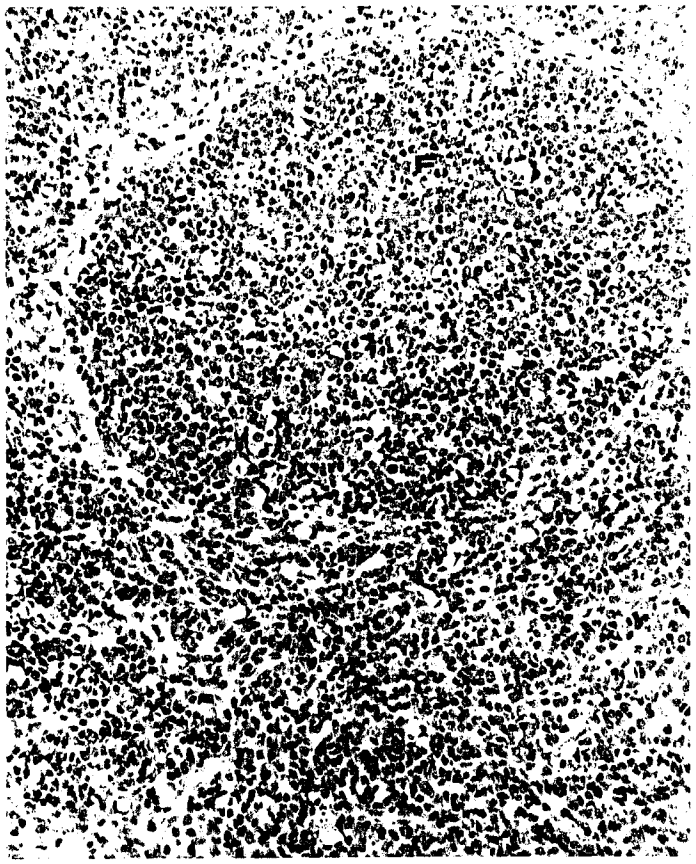

Fig 1 -- Lymph node from a B. abortus infected lamb. Sinuses are filled with foamy macrophages (M). H & E stain; X 160.

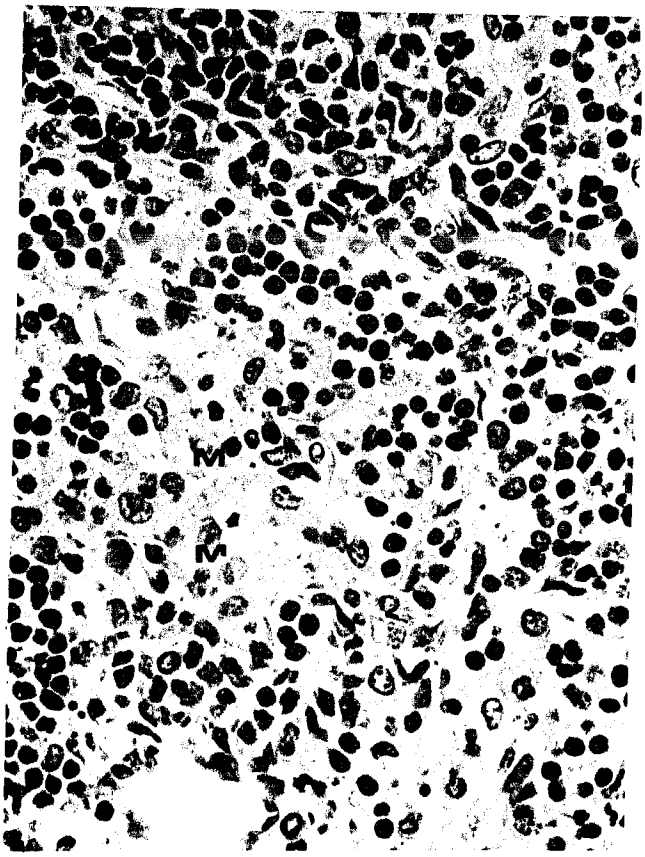

Fig 2 -- Cervical lymph node from a B. abortus infected lamb with a densely cellular cortex and a primary follicle (F) H & E stain; X 160.

filled with macrophages with abundant eosinophilic cytoplasm and a few lymphocytes, eosinophils and neutrophils.

The spleens of B. abortus infected lambs had numerous lymphoid follicles and a hypocellular red pulp. The red pulp was comprised of primarily plasma cells.

The thymus from control lambs had densely cellular cortices and the medullary connective tissue contained a few mononuclear and segmented leukocytes with large eosinophilic granules. In B. abortus infected lambs, thymic cortices were moderately depleted and the medulla contained a few mononulear and segmented leukocytes with large eosinophilic granules. Trabecular connective tissue contained a few band and mature neutrophils and moderate numbers of mononuclear, band and segmented leukocytes with large eosinophilic granules. No significant lesions were observed in the liver, lung and adrenal gland.

Hematology -- There was no significant ($P > 0.05$) difference in the hematological response between B. abortus infected and saline inoculated lambs. With the nonspecific esterase stain, monocytes stained bright red. Leukocyte differential counts obtained using the nonspecific esterase stain were similar to those obtained using the modified Wright's stain.

Serology -- All serum samples from saline inoculated controls were card test and complement fixation test negative for antibodies to B. abortus. All serum samples from B. abortus infected lamb 4 and serum samples taken from

B. abortus infected lambs 1, 2 and 3 on PI days 0, 2, 4 and 8 were card and complement fixation test negative for antibodies to B. abortus. At PI day 16, serum samples from B. abortus infected lambs 1, 2 and 3 were card test positive and complement fixation test positive for antibodies to B. abortus. Respectively, complement fixation titers in lambs 1, 2 and 3 were 1:41, 1:13 and 1:14.

Neutrophil Phagocytic Function -- Phagocytosis of B. abortus and S. aureus in autologous serum, fetal calf serum and Gey's solution by neutrophils from B. abortus infected and saline inoculated lambs was determined (Table 2), and the change from baseline percentage (Day 0) was calculated (Table 3). The phagocytosis of B. abortus by neutrophils from B. abortus infected and saline inoculated lambs in autologous serum was compared (Fig 3). The phagocytosis of B. abortus in autologous serum from B. abortus infected lambs was significantly ($P < 0.05$) higher than the phagocytosis of B. abortus in autologous serum for saline inoculated lambs. Using fetal calf serum, the phagocytosis of B. abortus in B. abortus infected lambs was significantly ($P < 0.05$) higher than the phagocytosis of B. abortus in saline inoculated lambs (Fig 4). There was no significant ($P > 0.05$) difference in the phagocytosis of B. abortus in Gey's solution in B. abortus infected and saline inoculated lambs.

The phagocytosis of S. aureus in autologous serum from B. abortus infected lambs was not significantly ($P > 0.05$)

Table 2 - The phagocytosis of *B. abortus* and *S. aureus* by neutrophils from *B. abortus* infected and saline inoculated neonatal sheep

PI Days Uninfected controls	(n)	Phagocytosis Percentage BA	BF	BG	SA	SF	SG
0	(3)	62	29	11	43	36	25
2	(3)	37	16	22	38	41	16
4	(3)	49	23	16	56	46	41
8	(3)	46	18	16	50	28	16
16	(3)	30	10	9	56	22	29
B. abortus infected							
0	(4)	25	0	0	64	28	16
2	(4)	51	3	1	88	56	52
4	(4)	45	1	0	83	40	24
8	(4)	53	46	23	82	47	47
16	(4)	64	26	4	88	54	21

PI Days = Days postinoculation, n = number of fetuses, B = *B. abortus*, A = autologous serum, F = fetal calf serum, G = Gey's solution, S = *S. aureus*.

Table 3 - The difference in phagocytosis of *B. abortus* and *S. aureus* by neutrophils after *B. abortus* infection and saline inoculation of neonatal sheep

PI Days		Percentage difference in phagocytosis					
Uninfected controls	(n)	BA	BF	BG	SA	SF	SG
0	(3)	0	0	0	0	0	0
2	(3)	-24	- 8	+10	-13	0	- 5
4	(3)	-13	- 4	+ 4	+ 6	+ 4	+16
8	(3)	-15	- 9	+ 4	+ 4	-14	- 7
16	(3)	-31	-17	- 4	+ 9	-20	+ 4
B. abortus infected							
0	(4)	0	0	0	0	0	0
2	(4)	+26	+ 3	+ 1	+28	+31	+34
4	(4)	+21	+ 1	0	+18	+14	+ 5
8	(4)	+30	+46	+23	+17	+17	-29
16	(4)	+40	+25	+ 4	+23	+28	+ 7

PI Days = Days postinoculation, n = number of fetuses, B = *B. abortus*, A = autologous serum, F = fetal calf serum, G = Gey's solution, S = *S. aureus*.

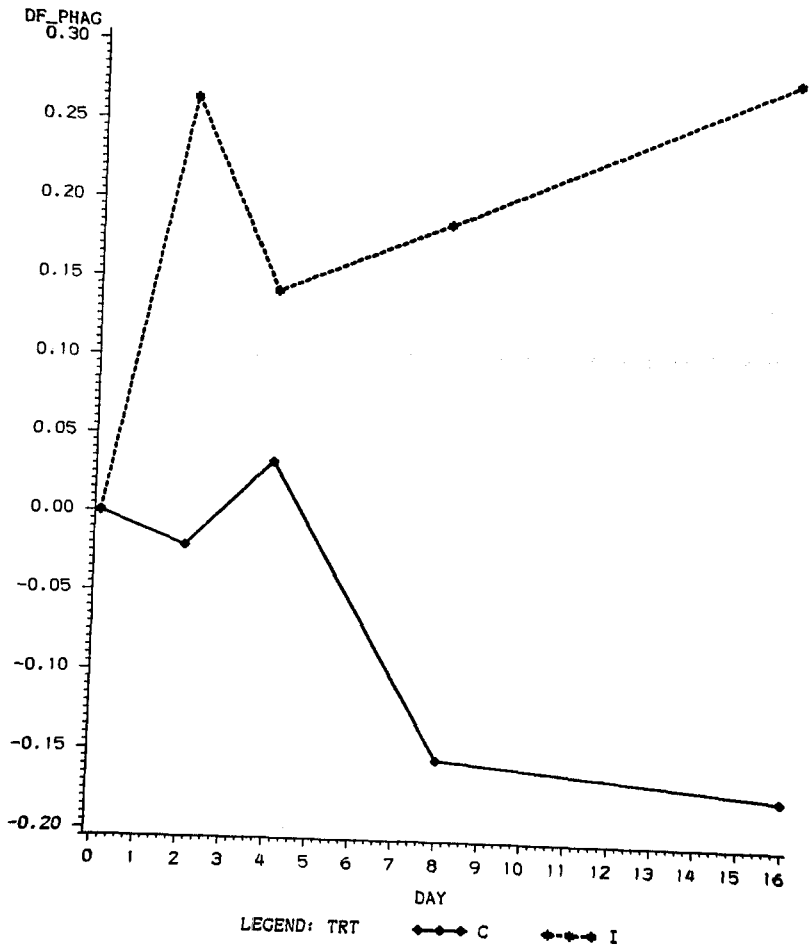

Fig 3 -- A comparison of change of phagocytosis (DF-Phag) by neutrophils from B. abortus infected lambs (I) and saline inoculated lambs (C) that phagocytosed B. abortus in autologous serum.

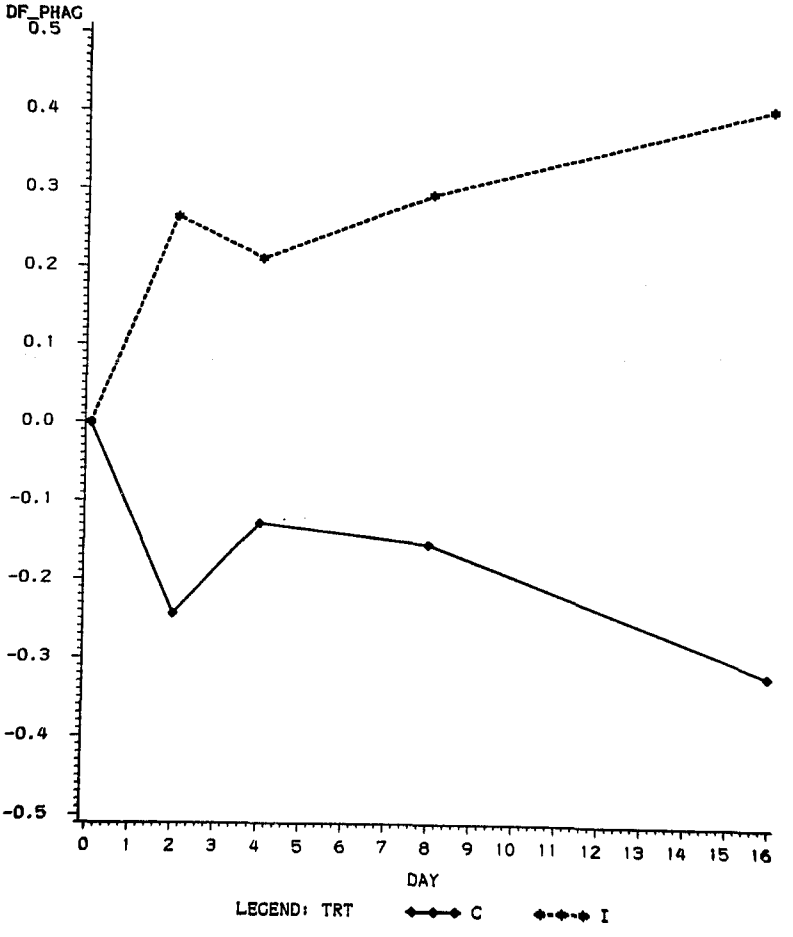

Fig 4 -- A comparison of phagocytosis (DF-Phag) by neutrophils from B. abortus infected lambs (I) and saline inoculated lambs (C) that phagocytosed B. abortus in fetal calf serum.

different from the phagocytosis of S. aureus in autologous serum from saline inoculated lambs. The phagocytosis of S. aureus in fetal calf serum and in Gey's solution by neutrophils from B. abortus infected lambs was significantly ($P < 0.05$) higher than the phagocytosis of S. aureus in fetal calf serum and in Gey's solution by neutrophils from saline inoculated lambs (Fig 5) and (Fig 6).

Neutrophil Killing Function -- Killing of B. abortus and S. aureus in autologous serum, fetal calf serum and Gey's solution by neutrophils from B. abortus infected and saline inoculated lambs was determined (Table 4), and the change from baseline percentage (Day 0) was calculated (Table 5). In autologous serum, the percentage of neutrophils from B. abortus infected lambs that contained killed B. abortus organisms was significantly ($P < 0.05$) greater than neutrophils from saline inoculated lambs that contained killed B. abortus organisms (Fig 7). In fetal calf serum and Gey's solution, neutrophils from saline inoculated lambs that contained killed B. abortus organisms was not significantly ($P > 0.05$) different from neutrophils from B. abortus infected lambs that contained killed B. abortus organisms.

Neutrophils from B. abortus infected lambs that contained killed S. aureus in autologous serum, fetal calf serum and Gey's solution was not significantly ($P > 0.05$) different from neutrophils from saline inoculated lambs that contained killed S. aureus organisms in

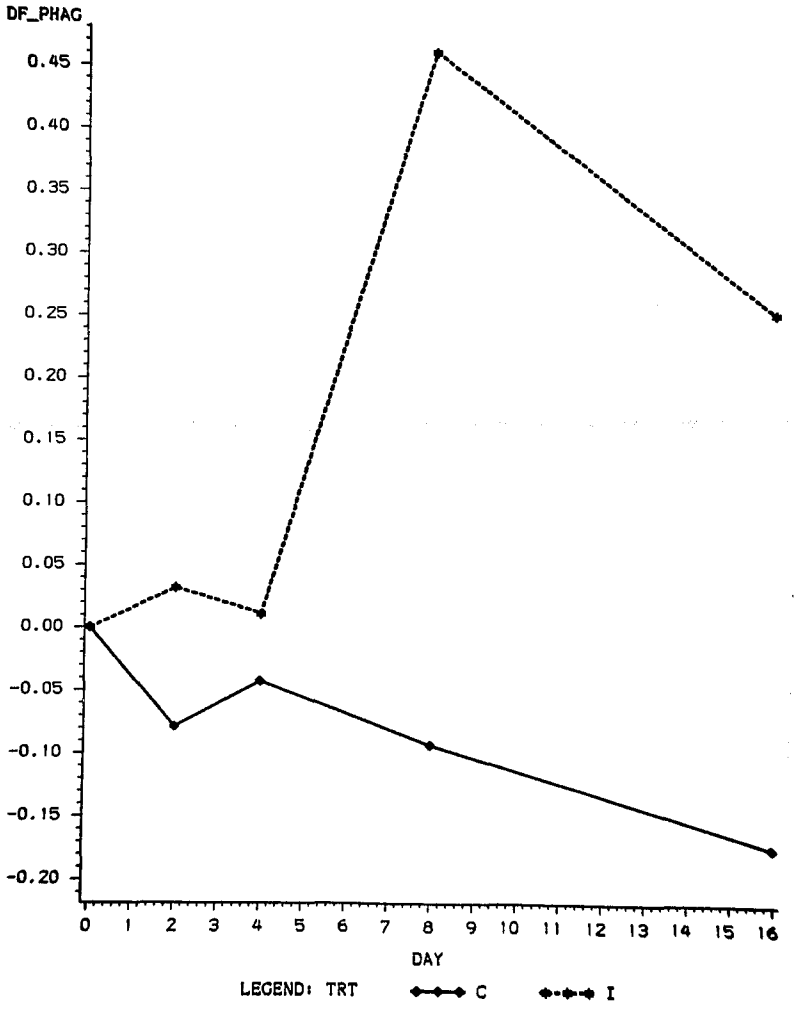

Fig 5 -- A comparison of phagocytosis (DF-Phag) by neutrophils from B. abortus infected lambs (I) and saline inoculated lambs (C) that phagocytosed S. aureus in fetal calf serum.

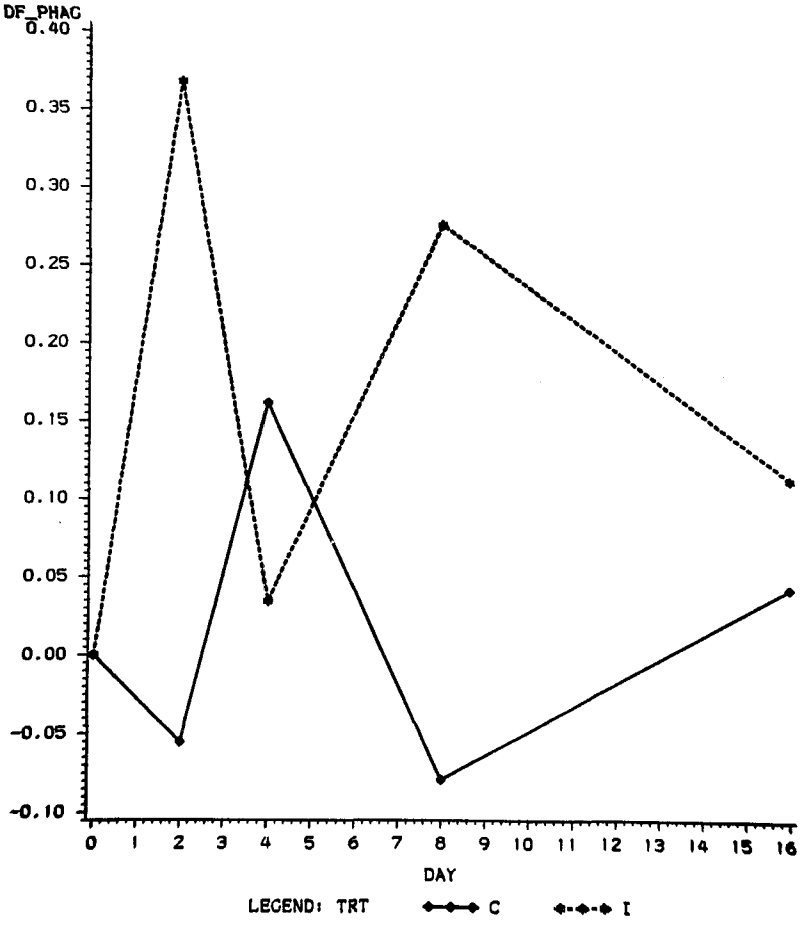

Fig 6 -- A comparison of phagocytosis (DF-Phag) by neutrophils from B. abortus infected lambs (I) and saline inoculated lambs (C) that phagocytosed S. aureus in Gey's solution.

Table 4 - The killing of *B. abortus* and *S. aureus* by neutrophils from *B. abortus* infected and saline inoculated neonatal sheep

PI Days Uninfected controls	(n)	Killing Percentage BA	BF	BG	SA	SF	SG
0	(3)	30	9	4	10	5	2
2	(3)	19	4	1	4	4	0
4	(3)	2	3	0	20	4	4
8	(3)	25	2	5	18	4	0
16	(3)	1	10	0	10	0	1
B. abortus infected							
0	(4)	3	0	0	6	2	2
2	(4)	38	13	0	13	12	10
4	(4)	15	0	0	3	1	0
8	(4)	18	17	5	11	21	2
16	(4)	52	7	0	8	2	2

PI Days = Days postinoculation, n = number of fetuses, B = *B. abortus*, A = autologous serum, F = fetal calf serum, G = Gey's solution, S = *S. aureus*.

Table 5 - The difference in killing of *B. abortus* and *S. aureus* by neutrophils after *B. abortus* infection and saline inoculation of neonatal sheep

PI Days Uninfected controls	(n)	Percentage difference in killing BA	BF	BG	SA	SF	SG
0	(3)	0	0	0	0	0	0
2	(3)	- 1	- 6	-11	- 8	- 5	0
4	(3)	-17	- 6	-15	+ 2	- 4	- 1
8	(3)	- 6	- 6	- 4	- 6	- 5	- 3
16	(3)	-17	- 4	-15	- 4	- 6	-29
B. abortus infected							
0	(4)	0	0	0	0	0	0
2	(4)	+15	+ 1	0	+ 7	+ 4	+ 5
4	(4)	+ 1	0	0	- 3	- 2	- 4
8	(4)	+ 7	+ 5	+19	+ 2	+ 7	- 1
16	(4)	+28	+ 2	0	+ 1	- 1	0

PI Days = Days postinoculation, n = number of fetuses, B = *B. abortus*, A = autologous serum, F = fetal calf serum, G = Gey's solution, S = *S. aureus*.

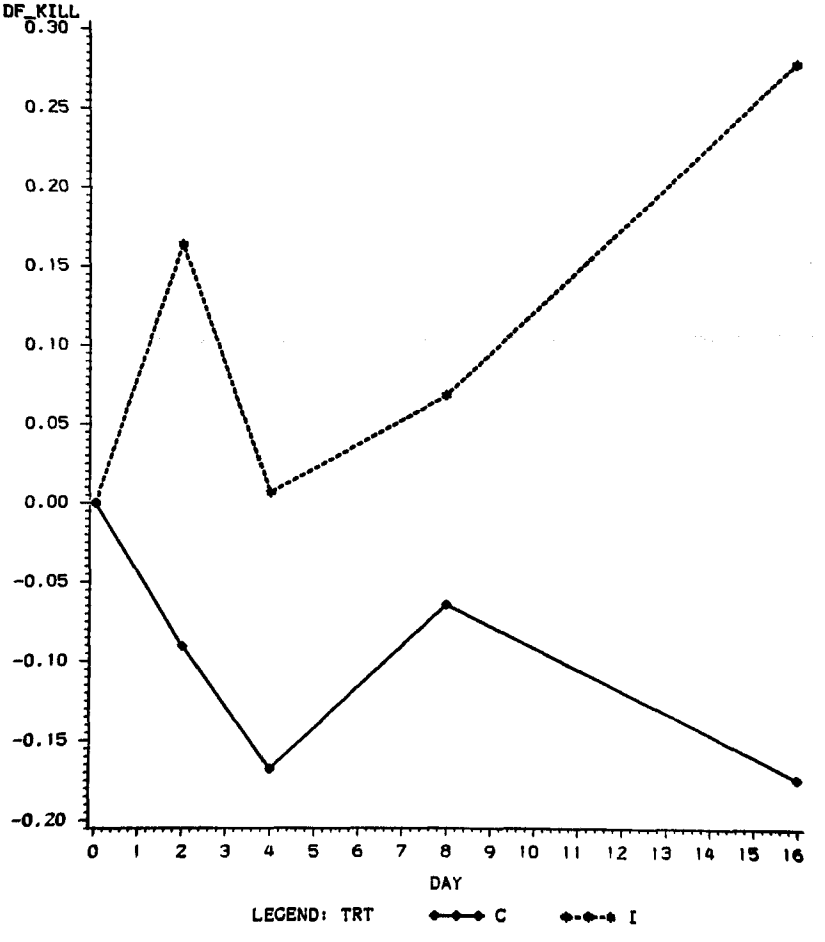

Fig 7 -- A comparison of killing (DF Kill) by neutrophils from B. abortus infected lambs (I) and saline inoculated lambs (C) that contained killed B. abortus in autologous serum.

autologous serum, fetal calf serum and Gey's solution.

Discussion

Although B. abortus was isolated from several organs, gross and microscopic lesions were only observed in the regional lymph nodes. The morphologic lesions were consistent with and similar to findings in B. abortus infected cattle.[4]

The reasons for increased neutrophil phagocytic and killing functions after B. abortus infection in this study were not determined. Antibodies enhance neutrophil phagocytosis by opsonizing the antigen and some antibodies alone are capable of killing bacteria.[94,95] While antibodies may have been responsible for the increased phagocytosis and killing by neutrophil from B. abortus infected lambs in autologous serum at PI day 16, antibodies were not present at PI days 0, 2, 4 and 8 and could not have accounted for increased phagocytosis and killing before PI day 16. Macrophage phagocytosis of nonbacterial particulate matter is increased by fibronectin which is a high molecular weight opsonic protein present in acute inflammatory responses.[96] In the presence of serum, fibronectin was reported to increase neutrophil phagocytosis of S. aureus.[97] Since fibronectins can be rapidly produced (1 day after infection) their presence in autologous serum may possibly explain the increased phagocytosis of B. abortus in

autologous serum at PI days 2, 4 and 8.

Neutrophil phagocytosis, killing and chemotaxis are enhanced during active bacterial infections.[74, 98-101]. It has been suggested that chemotactic factors interact with neutrophils and increase neutrophil metabolic and functional abilities.[101,102] Such a mechanism would explain the increased functional abilities of neutrophils from B. abortus infected lambs used in this study.

The isolation of B. abortus from several organs of all B. abortus infected lambs is proof that the organisms persist at least 17 days in infected neonatal sheep. How the B. abortus infection is eventually eliminated in most animals infected in utero or as neonates and why neonates are more resistent to B. abortus infection than adults remain undetermined.

Similarities in morphologic lesions and immunological responses in B. abortus infected lambs and cattle suggest that the lamb may serve as a reliable experimental model for bovine brucellosis.

Chapter IV

Morphologic Lesions, Hematological and Immunological Responses and Neutrophil Functions in Adult Sheep Infected with Brucella abortus

Introduction

Normal adult cattle are resistent to many infectious organisms. Most infectious agents stimulate humoral, cell mediated and phagocytic responses. In most instances, these properties completely eliminate the parasite from the host.[65-67] However, in B. abortus infections, organisms tend to localize and persist in the mammary gland and lymph nodes.[1-3,12,26,27] This is in contrast to B. abortus infection in calves. In calves, brucella organisms are usually completely eliminated from the host and seldom localize in the mammary gland or lymph node.[22] In a few instances, in utero or neonatal B. abortus infected animals become serologic positive and shed organisms immediately prior or after abortion or parturition (latently infected animals).[23,24] The greater resistance of neonatal calves to B. abortus localization and persistence is due to undetermined resistance factors.

The use of cattle in bovine brucellosis research is expensive and complicated by housing requirements, space and technical difficulties (handling and collection of samples). Hence there is need of a closely related, less expensive,

smaller, easier to handle, B. abortus susceptible experimental model. Sheep can be naturally and experimentally infected with B. abortus, and many aspects of the natural and experimental disease in sheep mimic bovine brucellosis.[30,32,37] They are less expensive, require smaller housing units and present fewer technical problems than cattle. The purposes of this study were: to evaluate adult sheep as an experimental model for bovine brucellosis and; to determine the role of nonspecific host resistance in B. abortus infection.

Materials and Methods

Experimental Design -- Two ewes were infected with B. abortus and 2 ewes were inoculated with saline. Blood and serum samples were collected before inoculation and at postinoculation (PI) days 2, 4, 8 and 16. All ewes were sacrified at PI day 17.

Animals -- Four adult, non-pregnant ewes were obtained from the Louisiana State University sheep farm. Animals were housed on a roofed, open slab with a concrete floor. They were fed hay and water. All animals were negative for antibodies to B. abortus by the card test (buffered brucella antigen) and complement fixation test.

Two ewes were used as saline inoculated controls and 2 ewes were inoculated with B. abortus Strain 2308 (4×10^6 organisms) in the dorsal neck muscles. Blood was collected

in dipotassium ethylenediaminotetraacetate (EDTA) tubes and siliconized glass tubes at PI days 2, 4, 8 and 16. Blood collected in EDTA was used in neutrophil function tests, and to obtain total and differential leukocyte counts. Serum from blood collected in siliconized tubes was used in brucella serologic tests and as autologous serum in neutrophil function tests. Tissues were collected for culture and for histologic evaluation. Tissues collected for histologic evaluation were fixed in 10 percent 4°C formalin.

Culture Procedures -- The tissues selected included lung, kidney, spleen, liver, intestinal lymph node, and cervical lymph node which were collected using aseptic technique for bacteriological analysis. Tissues were cultured immediately or stored overnight at 4°C and cultured within 24 hours of harvest.

Tissues were prepared for culturing by cutting into 2-3 mm pieces with a sterile surgical blade. Tissue were placed in a sterile blender with 10 to 30 ml of sterile saline. The tissue saline mixture was blended for 20-30 seconds. Minced tissues were streaked on Tryptic Soy agar plates[a] with 5 percent heat-inactivated horse serum and brucella selective media [81] (with the addition of 0.2 g L cysteine, 1:5000 and 0.25 gm erythritol, 1:4000 per liter). Plates were incubated for 2-5 days in a 37°C incubator.

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[a]Difco Laboratories, Detroit, Michigan.

B. abortus was identified as small round, pale-honey colored colonies. These bacteria were coccobacilli, Gram stain negative, urease and oxidase positive.

Bacteria -- Stock cultures of Staphylococcus aureus Strain 520A[b] were stored at 4°C on Tryptose Soy agar. For daily use, stock S. aureus was inoculated in 5-10 ml of Tryptose broth and incubated 16-20 hours at 37° C. Bacteria were washed 3 times in physiologic Gey's solution, centrifuged at 1600 x g for 10 minutes at 4°C and the pellet was suspended in 5 ml of physiologic Gey's solution. The absorbance reading was adjusted to .20 - .25 optical density (OD).[c] Bacteria were cultured on Tryptose Soy agar plates for determination of the number of bacteria per ml. The microdrop plating method was used for this determination.

B. abortus Strain 2308[d] stock was maintained in sterile saline. Stock B. abortus was inoculated in Tryptic Soy broth and incubated at 37°C for 16-20 hours. Bacteria were washed 3 times in physiologic Gey's solution, centrifuged at 1600 x g for 10 minutes at 4°C and the pellet was suspended in 5 ml of physiologic Gey's solution. The absorbance level was adjusted to .02 and .04 OD. Bacteria were cultured on Tryptic Soy agar plates enriched with 5

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[b]Dr. H. Cox, Department of Microbiology and Parasitology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803.
[c]Spectronic 20 Spectrophotometers, Bausch and Lomb, Inc., Rochester, New York.
[d]Dr. B. L. Deyoe, National Animal Disease Center, Agriculture Research Service, United States Department of Agriculture, Ames, Iowa 50010.

percent heat-inactivated horse serum for determination of the number of bacteria per ml. The microdrop plating method was used for this determination.

Tissue Processing -- Freshly collected sections of liver, lung, spleen, kidney, intestinal lymph node and cervical lymph node were fixed at 10 percent formalin at 4° C. Tissues were stored in cold formalin.

Tissues were embedded in plastic as described.[82] A 5 g pack of white powder, benzoyl peroxide (catalyst), was added to 5 g of a liquid, 2-butoxy ethanol (solution B), and mixed with a magnetic stirrer. Infiltrating solution was prepared by mixing 5 ml of solution B with 50 ml of hydroxy ethyl methacrylate (solution A). Sections of tissues were added to this infiltrating solution and placed in a vacuum dessicator. A vacuum was established and maintained at 4°C for 24 hours. After 24 hours the infiltrating solution was changed and another vacuum was created and maintained at 4°C for an additional 24 hours.

The embedding solution was prepared by mixing 50 ml of solution A with 5 ml of solution B and 2 ml of N-N-dimethyl aniline (solution C). Two to three ml aliquots were pipetted into the lower depressions of plastic molds. Infiltrated tissue specimens were placed in the molds and covered with the embedding solution. The molds were placed in a vacuum dessicator. A vacuum was created and the vacuum dessicator was maintained at 4°C for 48 hours to allow polymerization.

Using a microtome[e] with a glass knife holder, plastic embedded tissues were cut in 2 micron sections with a glass knife. Sections were stained with hematoxylin and eosin. Selected tissues were stained with nonspecific esterase.

Hematology -- Blood was collected in EDTA tubes. This blood was used for preparation of blood smears. Total leukocyte counts were obtained with an electronic cell counter.[f] Blood smears were stained with a modified Wright's stain.[g] All blood smears were examined with an oil immersion lens for differential cell counts. Additionally, blood smears were stained for nonspecific esterase after fixation for 60 seconds in a pH 6.16 fixative composed of: KH_2PO_4, 100 mg; distilled H_2O, 30 ml; acetone, 45 ml; and formalin (30%), 25 ml.[83] Additionally, nonspecific esterase staining was obtained by incubating fixed blood smears in a solution composed of: Sorenson's phosphate buffer, 44.5 ml; hexazolized pararosaniline, 0.25 ml; and alpha-naphthyl butyrate solution, 3ml. Incubation was for 45 minutes in a 37° C water bath. Smears were washed in distilled water and counter stained in a 0.5 percent methyl green solution for 1 minute. Smears were then washed 3 times in distilled water. They were air dried and coverslipped. All smears were

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[e]Sorvall Porter-Blum Microtome, Ivan Sorvall Incorporated, Newtown, Connecticut 06470.
[f]Houlter Electronics Incorporated, Hialeah, Florida.
[g]Diff Quik, American Scientific Products, Division of American Hospital Supply Corporation, McGraw Park, Il.

They were air dried and coverslipped. All smears were examined with an oil immersion lens for differential cell counts and nonspecific esterase positive (monocytes) and negative cells.

Neutrophil Phagocytosis and Killing Assay -- Neutrophil phagocytosis and killing functions were assayed by modifications of Pantazis' method[84] using acridine orange.[h] Blood collected in EDTA was washed 2 times in physiologic saline and once in physiologic Gey's solution followed by centrifugation at 400 x g for 10 minutes at 4°C. Approximately 0.5 x 10^6 leukocytes (in 0.3 ml of pelleted blood) were mixed with 30 x 10^6 bacteria (S. aureus Strain 520A or B. abortus Strain 2308 suspended in 0.5 ml of physiologic Gey's solution) and 0.2 ml serum (autologous or fetal calf serum) or 0.2 ml physiologic Gey's solution. Six assay mixtures prepared on each sample included: pelleted blood and B. abortus mixed with 1) autologous serum, 2) fetal calf serum, 3) and physiologic Gey's solution, and pelleted blood and S. aureus mixed with, 4) autologous serum, 5) fetal calf serum, and 6) physiologic Gey's solution. The 6 samples were incubated for 60 minutes in a 37°C water bath that rotated 200 revolutions per minute. After incubation, all tubes were vortexed and centrifuged at 400 x g for 10 minutes at 4°C. All samples were stored at 4°C and examined within 4 hours.

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[h]Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, New Jersey, 07410.

The supernatant was removed and a drop of pelleted blood and a drop of 0.14 percent acridine orange was added to a clean glass slide and coverslipped. Cells were examined with a fluorescent microscope.[i] The 63X and the 100X oil immersion lens and an epifluorescent light source with a 510mm filter were used to examine wet mounts. Three replicates of 100 leukocytes were counted for each assay mixture. Neutrophils that ingested 1 or more bacterial organisms were considered phagocytic. Neutrophils that contained 1 or more dead (red) bacterial organisms were counted as neutrophils which had killed bacteria.

Calculations of phagocytytic and killing functions were obtained from 2 formulas:

$$\text{Phagocytosis \%} = \frac{\text{neutrophils ingesting 1 or more oganisms}}{\text{total neutrophils counted}} \times 100$$

$$\text{Killing \%} = \frac{\text{neutrophils containing 1 or more dead organisms}}{\text{total neutrophils ingesting bacteria}} \times 100$$

Baseline phagocytic and killing levels were obtained in day 0 ewes (before saline or B. abortus inoculation). The change in neutrophils that phagocytosed and killed B. abortus and S. aureus after B. abortus infection was compared with the change in neutrophils that phagocytosed

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[i]Carl Zeiss, D 7082 Oberkochen, West Germany.

and killed B. abortus and S. aureus after saline inoculation.

Serology -- The card (buffered brucella antigen)[14] and the complement fixation test were used to detect brucella specific antibodies.[14,61]

Statistical Analysis -- Data were evaluated by analysis of variance with the Duncan's Multiple Range test for means separation.

RESULTS

Culture - At the time of sacrifice, PI day 17, both uninfected control ewes were negative for B. abortus and both B. abortus infected ewes were positive for B. abortus. by bacteriological culture. B. abortus was isolated only from the liver of both B. abortus infected ewes.

Pathology -- Gross abnormalities were limited to marked diffuse enlargement of the cervical lymph node of B. abortus infected ewe 3185.

Cervical lymph nodes from both control sheep were characterized by moderately thick cortices and moderately cellular sinuses and medullary cords. Cortices contained a few secondary follicles and moderate numbers of lymphocytes. Sinuses contained foamy macrophages with abundant eosinophilic cytoplasm, a few lymphocytes and occasional neutrophils and eosinophils. Medullary cords were populated by moderate numbers of lymphocytes, a few plasma cells and

occasional eosinophils.

Cervical lymph nodes from infected sheep had moderately thickened cortices with numerous prominent secondary lymphoid follicles (Fig 1) and moderately thickened paracortical areas. Medullary cords and subcapsular sinuses were slightly to markedly hypercellular. Small to moderate size areas around secondary follicles were frequently populated by dense accumulations of plasma cells. Medullary cords contained numerous plasma cells and foci of few to moderate numbers of mononuclear and segmented leukocytes with large eosinophilic granules (Fig 2). Sinuses contained slightly to moderately increased numbers of macrophages.

Spleens from both infected ewes had moderately hypocellular red pulp and prominent lymphoid follicles. In the red pulp there were a few plasma cells and prominent reticulum cells. No significant lesions were present in the liver, lung, kidney and adrenal gland.

Hematology -- There was no significant ($P > 0.05$) difference in the hematological responses in B. abortus infected ewes and saline inoculated ewes. With the nonspecific esterase stain, monocytes stained bright red. Leukocyte differential counts obtained using the nonspecific esterase stain were similar to those obtained using the modified Wright's stain.

Serology -- All serum samples from both saline inoculated ewes and the PI days 0, 2, and 4 serum samples

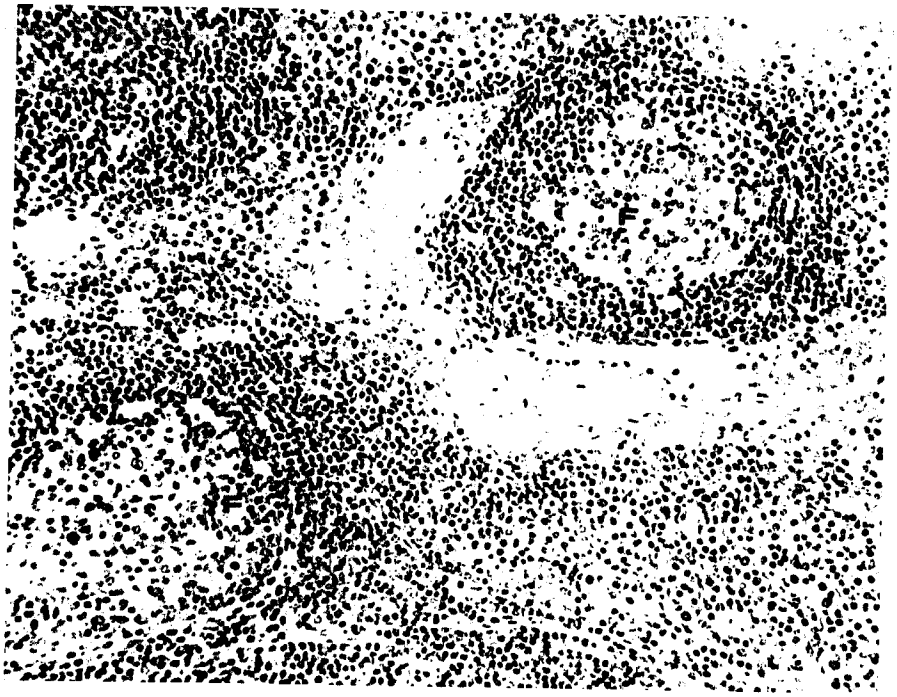

Fig 1 -- Lymph node from a B. abortus infected ewe. There are prominent secondary lymphoid follicles (F). H & E stains; X 160.

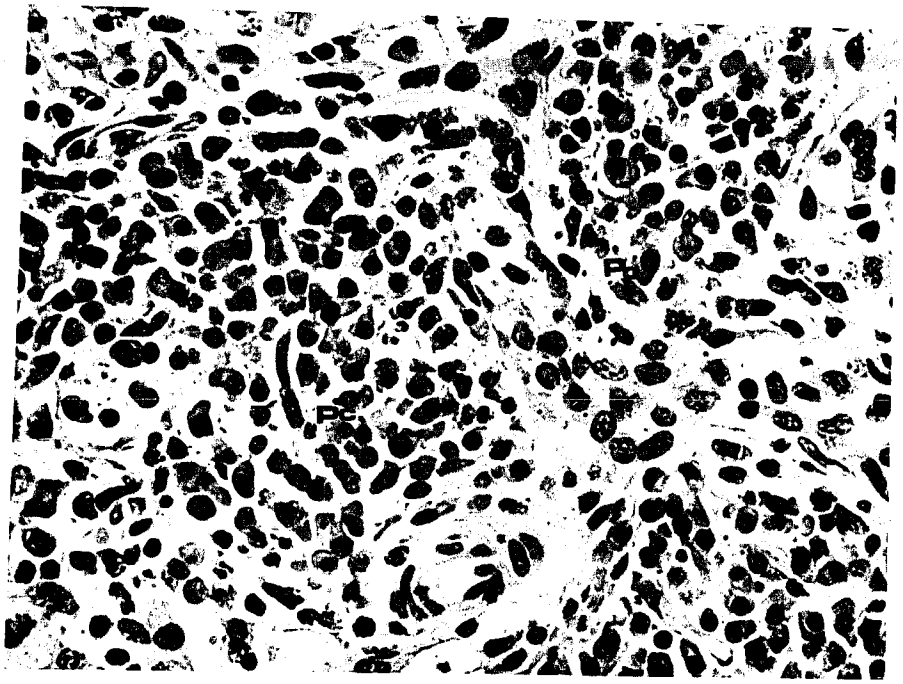

Fig 2 -- Lymph node from a B. abortus infected ewe. The medullary cords contain numerous plasma cells (PC). H & E stains; X 400.

from both B. abortus infected ewes were card test and complement fixation test negative for antibodies to B. abortus. Postinoculation day 8 and 16 serum samples from B. abortus infected ewes were card test positive for antibodies to B. abortus. The PI day 16 serum sample from one B. abortus infected ewe had a complement fixation titer of 1:41.

Neutrophil Phagocytic Function -- Phagocytosis of B. abortus and S. aureus in autologous serum, fetal calf serum and Gey's solution by neutrophils from B. abortus infected and saline inoculated ewes was determined (Table 1), and the change from baseline percentage (Day 0) was calculated (Table 2). The phagocytosis of B. abortus in autologous serum, fetal calf serum and Gey's solution was not significantly ($P > 0.05$) different from the phagocytosis of B. abortus in autologous serum, fetal calf serum and Gey's solution in B. abortus infected ewes. The phagocytosis of S. aureus in autologous serum, fetal calf serum and Gey's solution in saline inoculated ewes was not significantly ($P > 0.05$) different from the phagocytosis of S. aureus in autologous serum, fetal calf serum and Gey's solution in B. abortus infected ewes.

Neutrophil Killing Function -- Killing of B. abortus and S. aureus in autologous serum, fetal calf serum and Gey'solution by neutrophils from B. abortus infected and saline inoculated ewes was determined (Table 3), and the change from baseline percentage (Day 0) was calculated

Table 1 - The phagocytosis of *B. abortus* and *S. aureus* by neutrophils from *B. abortus* infected and saline inoculated adult sheep

PI Days Uninfected controls	(n)	Phagocytosis Percentage BA	BF	BG	SA	SF	SG
0	(2)	35	34	15	59	58	41
2	(2)	37	16	5	72	72	56
4	(2)	27	21	11	65	69	67
8	(2)	28	21	8	64	78	47
16	(2)	42	39	23	75	74	51
B. abortus infected							
0	(2)	33	14	7	77	77	54
2	(2)	58	21	15	87	83	67
4	(2)	56	34	17	90	73	64
8	(2)	62	24	22	80	70	51
16	(2)	57	7	9	55	64	48

PI Days = Days postinoculation, n = number of fetuses, B = *B. abortus*, A = autologous serum, F = fetal calf serum, G = Gey's solution, S = *S. aureus*.

Table 2 - The difference in phagocytosis of *B. abortus* and *S. aureus* by neutrophils after *B. abortus* infection and saline inoculation of adult sheep

PI Days		Percentage difference of phagocytosis					
Uninfected controls	(n)	BA	BF	BG	SA	SF	SG
0	(2)	0	0	0	0	0	0
2	(2)	+ 2	-18	-10	+13	+13	+15
4	(2)	- 8	-13	- 4	+ 6	+ 9	+26
8	(2)	- 7	-13	- 7	+ 5	+19	+ 6
16	(2)	+ 7	+ 5	+ 8	+16	+15	+10
B. abortus infected							
0	(2)	0	0	0	0	0	0
2	(2)	+25	+ 7	+ 8	+10	+ 5	+13
4	(2)	+23	+20	+10	+13	- 4	+10
8	(2)	+29	+10	+15	+ 3	- 7	- 3
16	(2)	+24	- 7	+ 2	-22	-13	- 6

PI Days = Days postinoculation, n = number of fetuses, B = *B. abortus*, A = autologous serum, F = fetal calf serum, G = Gey's solution, S = *S. aureus*.

Table 3 - The killing of *B. abortus* and *S. aureus* by neutrophils from *B. abortus* infected and saline inoculated adult sheep

PI Days Uninfected controls	(n)	Killing Percentage BA	BF	BG	SA	SF	SG
0	(2)	6	2	1	5	3	1
2	(2)	20	9	20	29	17	31
4	(2)	26	0	0	34	11	7
8	(2)	21	14	8	20	7	3
16	(2)	23	11	4	23	4	3
B. abortus infected							
0	(2)	9	0	0	13	8	6
2	(2)	28	22	6	17	8	2
4	(2)	64	19	3	30	11	5
8	(2)	33	18	0	19	2	0
16	(2)	60	0	0	26	0	0

PI Days = Days postinoculation, n = number of fetuses, B = *B. abortus*, A = autologous serum, F = fetal calf serum, G = Gey's solution, S = *S. aureus*.

Table 4 - The difference in killing of *B. abortus* and *S. aureus* by neutrophils after *B. abortus* infection and saline inoculation of adult sheep

PI Days		Percentage difference in killing					
Uninfected controls	(n)	BA	BF	BG	SA	SF	SG
0	(2)	0	0	0	0	0	0
2	(2)	+14	+ 7	+19	+24	+14	+30
4	(2)	+20	- 2	- 1	+29	+ 8	+ 6
8	(2)	+15	+12	+ 7	+15	+ 4	+ 2
16	(2)	+17	+ 9	+ 3	+18	+ 1	+ 2
B. abortus infected							
0	(2)	0	0	0	0	0	0
2	(2)	+19	+22	+ 6	+14	0	- 4
4	(2)	+55	+19	+ 3	+ 7	+ 3	- 1
8	(2)	+24	+18	0	+ 6	- 6	- 6
16	(2)	+41	0	0	+13	- 8	- 6

PI Days = Days postinoculation, n = number of fetuses, B = *B. abortus*, A = autologous serum, F = fetal calf serum, G = Gey's solution, S = *S. aureus*.

(Table 4). Neutrophils from B. abortus infected ewes that contained killed B. abortus organism in autologous serum, fetal calf serum and Gey's solution was not significantly ($P > 0.05$) different from neutrophils from saline inoculated ewes that contained killed B. abortus organisms in autologous serum, fetal calf serum and Gey's solution. In autologous serum, fetal calf serum and Gey's solution neutrophils from saline inoculated ewes that contained killed S. aureus organisms was not significantly ($P > 0.05$) different from neutrophils from B. abortus infected ewes that contained killed S. aureus organisms in autologous serum, fetal calf serum and Gey's solution.

Discussion

The isolation of B. abortus from only the liver of both B. abortus infected ewes suggest that B. abortus may localize in the liver of non-pregnant ewes. The restriction of gross and microscopic lesions to regional lymph nodes was evidence that B. abortus was mildly pathogenic in ewes. Similar morphologic lesions were reported in B. abortus infected pregnant cattle.[4]

The hematological response in B. abortus infected ewes was not significantly different from the hematological response in saline inoculated ewes. The statistically insignificant increase in the percentage of neutrophils from B. abortus infected ewes to phagocytose and kill B. abortus

in autologous serum suggests that B. abortus specific antibody (present at PI days 8 and 16) opsonization did not enhance neutrophil phagocytosis and killing of B. abortus. Similar findings have been reported in adult B. abortus infected guinea pigs.[70]

In B. abortus infected ewes employed in this study, morphologic lesions and immunological responses were similar to changes observed in adult B. abortus infected cattle. These findings suggest that adult non-pregnant ewes may serve as adequate experimental models for brucellosis in non-pregnant cattle.

Chapter V

Summary and Conclusion

The morphologic lesions in neonatal and adult sheep infected with B. abortus were similar to morphologic lesions in cattle infected with B. abortus. Infected neonatal and adult sheep had moderately to markedly enlarged regional lymph nodes with prominent lymphoid follicles and medullary cords populated by numerous plasma cells. Granulomas frequently observed in lymph nodes of B. abortus infected cattle were not observed in these B. abortus infected sheep. This may be a consequence of time as these sheep lesions were observed at postinoculation (PI) day 17 and cattle were chronically infected. The relatively mild sheep lesions also suggest that they are less susceptible to B. abortus infection than cattle.

The bronchopneumonia, reactive lymph nodes and fibrin covered thoracic organs present in B. abortus infected ovines were similar to but more severe and occured faster than in infected bovine fetuses. These findings maybe resultant of route and dose of inoculation or may suggest that fetal sheep are more susceptible to B. abortus infection than fetal cattle.

Hematological responses in infected neonatal and adult sheep were not significantly different from the hematological responses in control neonatal and adult sheep.

Fetal sheep responded to B. abortus infection with a neutrophilia, lymphopenia and occasional monocytosis and band neutrophilia. The fetal hematological responses were possibly resultant of stress of B. abortus infection, hematological responses to B. abortus infection and/or bacterial endotoxins.

Adult and neonatal sheep produced antibrucella antibodies as early as PI day 8. Fetal sheep (examined up to PI day 6) did not produce antibrucella antibodies, however, some B. abortus infected fetuses had trace levels of immunoglobulins. The neonatal and adult sheep antibody responses were similar to those reported in cattle. The trace levels of immunoglobulins observed in B. abortus infected fetuses suggests that there was a primitive attempt to respond to the B. abortus infection.

Fetal cortisol levels were markedly elevated by B. abortus infection. This was probably due to the stress of B. abortus infection.

Neutrophil functional abilities were significantly increased after B. abortus infection in fetal and neonatal sheep but not in adult sheep. Since neutrophil functional abilities were present before antibrucella antibodies were produced, enhanced opsonization was not responsible for increased neutrophil functions. Nonspecific serum factors, increased neutrophil functional abilities, neutrophil activation by unknown serum factors, and fibronectin are possibly responsible for the increased neutrophil functional

abilities. Factors responsible for increased neutrophil functions were produced rapidly as increased neutrophil functional abilities were present at PI day 1 in fetal sheep and at PI day 2 in neonatal sheep.

Premature termination of pregnancy was observed in 6 of 12 B. abortus infected ovine fetuses. Five of these 6 fetuses were dead in utero at the time of collection and one was born alive. Premature pregnancy termination is a frequent finding in B. abortus infected pregnant cattle.

In conclusion, neutrophil functional abilities were increased by B. abortus infection in fetal and neonatal sheep and fetal serum cortisol levels were markedly increased by B. abortus infection. Additionally, because of similarities in morphologic lesions, immunological responses and the premature termination of pregnancy in sheep and cattle, sheep may serve as an adequate experimental model for bovine brucellosis.

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Responsibilities: 1) Supervision of necropsies by Year IV veterinary students, 2) Rendering gross and microscopic diagnosis on necropsies and surgical biopsies, 3) Consultation with referring veterinarians, 4) Selection of tissues for Year II gross tissue laboratory, 5) Teaching gross and microscopic pathology to Year II

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EXAMINATION AND THESIS REPORT

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Title of Thesis: Morphologic Lesions, Hematological and Immunological Responses and Neutrophil Functions in Fetal, Neonatal and Adult Sheep Infected with Brucella Abortus

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